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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
23 May 2002 (23.05.2002)

PCT

(10) International Publication Number
WO 02/40650 A2

- (51) International Patent Classification⁷: **C12N 9/00**
- (21) International Application Number: **PCT/EP01/12857**
- (22) International Filing Date:
7 November 2001 (07.11.2001)
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
0027779.8 14 November 2000 (14.11.2000) **GB**
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- (81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/40650 A2

(54) Title: **METHODS FOR LARGE SCALE PRODUCTION OF RECOMBINANT DNA-DERIVED TPA OR K2S MOLECULES**

(57) Abstract: The invention belongs to the field of thrombolysis and of tissue plasminogen activator (tPA) derivative production in prokaryotic cells. The invention relates to methods for the production of a recombinant DNA-derived tPA, a variant thereof or a (Kringle 2 Serine) K2S molecule or a variant thereof in prokaryotic cells, wherein said tPA or K2S or variant is secreted extracellularly as an active and correctly folded protein, and the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA or K2S or variant operably linked to the DNA coding for the signal peptide OmpA. The invention further relates to specific K2S derivatives obtainable by said method. The invention further relates to said DNA molecules and the use of said DNA molecules in said methods.

Methods for large scale production of recombinant DNA-derived tPA or K2S molecules

The invention belongs to the field of thrombolysis and of tissue plasminogen activator (tPA) derivative production in prokaryotic cells.

The invention relates to methods for the production of a recombinant DNA-derived tPA, a variant thereof or a (Kringle 2 Serine) K2S molecule or a variant thereof in prokaryotic cells, wherein said tPA or K2S or variant is secreted extracellularly as an active and correctly folded protein, and the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA or K2S or variant operably linked to the DNA coding for the signal peptide OmpA. The invention further relates to specific K2S derivatives obtainable by said method. The invention further relates to said DNA molecules and the use of said DNA molecules in said methods.

Background art

Tissue plasminogen activator (tPA) is a polypeptide containing 527 amino acid residues (27) with a molecular mass of 72 kDa. The molecule is divided into five structural domains. Nearby the N-terminal region is a looped finger domain, which is followed by a growth factor domain. Two similar domains, kringle 1 and kringle 2, are following. Both finger and kringle 2 domains bind specifically to the fibrin clots thereby accelerating tPA protein activation of bound plasminogen. Downstream of kringle 2 is the serine protease, with its catalytic site located at the C-terminus. The serine protease is responsible for converting plasminogen to plasmin a reaction important in the homeostasis of fibrin formation and clot dissolution. The correct folding of tPA requires the correct pairing of 17 disulfide bridges in the molecule (1).

Clinically, tPA is a thrombolytic agent of choice for the treatment of acute myocardial infarction, pulmonary embolism, stroke, peripheral arterial occlusions, and other thromboembolic diseases. It has the advantage of causing no side effects on systemic haemorrhaging and fibrinogen depletion (7). Bowes melanoma cells were first used as a source in tPA production for therapeutic purposes (12). Since a consistent process with efficient production of highly purified protein in good yield is required for clinical use, the construction of full-length recombinant-tPA (r-tPA) progressed to mammalian cells. Chinese hamster ovary cells were transfected with the tPA gene to synthesize the r-tPA (8, 22). The recombinant DNA derived product produced by a mammalian cell culture fermentation system is harvested and purified from the culture medium.

Attracted by simplicity and economy of production, a number of efforts in producing r-tPA from microorganisms, especially bacteria, and more especially from *Escherichia coli*, were investigated (10, 13, 30). Regarding the low yield and the formation of inclusion bodies, which resulted in misfolding and in an inactive enzyme, numerous strategies have been proposed to overcome these problems.

Several deletion-mutant variants including kringle 2 plus serine protease (K2S) were considered. However, the enzymatic activity of the recombinant-K2S (r-K2S) was obtained only when refolding processes of purified inclusion bodies from cytoplasmic compartment were achieved (16, 29). In order to avoid the cumbersome refolding processes, impurities of misfolded proteins, and periplasmic protein delivery, special bacterial expression systems were exploited (6, 31). Despite periplasmic expression of tPA, overexpression led to inactive aggregates, even in the relatively high oxidizing condition in the periplasm.

In the prior art, there are a few descriptions of methods for the preparation of recombinant K2S in *E. coli*. However, there is no disclosure of a method leading to a cost effective method for large scale production of biologically active K2S.

Obukowicz et al. (25) expressed and purified r-K2S from periplasmic space. The obvious disadvantage of this method was an extra periplasmic extraction step, which is not suitable for large scale production.

Saito et al. (29) disclose the cytoplasmic expression of r-K2S. The authors used an in vivo renaturation processes for the expressed r-K2S, which was purified from the cytoplasmic space of *E. coli* as inclusion body. Boehringer Mannheim use a similar cumbersome denaturing/refolding process involving the steps of cell digestion, solubilization under denaturing and reducing conditions and reactivation under oxidizing conditions in the presence of GSH/GSSG which is not cost effective (24) and requires mutation of the amino acid sequence with possibly antigenic potential.

In 1991, Waldenström et al. (34) constructed a vector (pEZZK2P) for the secretion of kringle 2 plus serine protease domain to *E. coli* culture supernatant. Hydroxylamine was used to remove the ZZ fusion peptide from IgG-Sepharose purified fraction. The cleavage agent hydroxylamine required modification of the cleavage sites of kringle 2 plus serine protease (Asn¹⁷⁷ → Ser and Asn¹⁸⁴ → Gln) thus to protect it from hydroxylamine digestion. However, the resulting non-native, not properly folded K2S molecule is not suitable for therapeutic purposes. No enzymatic

activity regarding fibrin binding/protease activity was disclosed. The unusual sequence may even activate the human immune system.

The problem underlying the present invention was thus to provide a commercially applicable method for large scale production of tPA molecules and derivatives thereof, e.g. K2S, wherein the K2S molecule is secreted in its biologically active form into the culture supernatant.

Description of the invention

The problem was solved within the scope of the claims and specification of the present invention.

10 The use of the singular or plural in the claims or specification is in no way intended to be limiting and also includes the other form.

The invention relates to a method for the production of a recombinant DNA-derived tissue plasminogen activator (tPA), a tPA variant, a Kringle 2 Serine protease molecule (K2S) or a K2S variant in prokaryotic cells, wherein said tPA, tPA variant, K2S molecule or K2S variant is
15 secreted extracellularly as an active and correctly folded protein, characterized in that the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S variant operably linked to the DNA coding for the signal peptide OmpA or a functional derivative thereof.

Surprisingly, the use of the signal peptide OmpA alone and/ or in combination with the N-
20 terminal amino acids SEGN (SEQ ID NO:9) / SEGNSD (SEQ ID NO:10) translocate the recombinant DNA-derived tPA, tPA variant, K2S molecule or K2S variant to the outer surface and facilitates the release of the functional and active molecule into the culture medium to a greater extent than any other method in the prior art. Before crossing the outer membrane, the recombinant DNA-derived protein is correctly folded according to the method of the present
25 invention. The signal peptide is cleaved off to produce a mature molecule. Surprisingly, the efficiency of signal peptide removal is very high and leads to correct folding of the recombinant DNA-derived protein.

Said signal peptide OmpA interacts with SecE and is delivered across the inner membrane by energy generated by SecA, which binds to Sec components (SecE-SecY). SecY forms a secretion
30 pore to dispatch the recombinant DNA-derived protein according to the invention. The space between the outer membrane and inner membrane of Gram-negative bacteria, periplasm, has higher oxidative condition in comparison to the cytoplasmic space. This supports the formation

of disulfide bonds and properly folding of the recombinant DNA-derived protein (e.g. K2S) in the periplasm to yield an active molecule. According to the present invention, the signal peptide will be cleaved off to produce a mature molecule. The complex of GspD secretin and GspS lipoprotein on the outer membrane serves as gate channel for secreting the recombinant DNA-derived protein according to the invention to the extracellular medium. This secretion process requires energy, which is generated in cytoplasm by GspE nucleotide-binding protein then transferred to the inner membrane protein (Gsp G-J, F and K-N). GspC transfers the energy to GspD by forming a cross-linker between a set of inner membrane protein (Gsp G-J, F and K-N) and GspD. Before crossing the outer membrane successfully, the recombinant DNA-derived protein is correctly folded.

Operably linked according to the invention means that the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant (preferably comprising the nucleic acid encoding SEGN or SEGNSD at its N-terminal portion) is cloned in close proximity to the OmpA DNA into the vector in order to achieve expression of the OmpA-tPA, tPA variant, K2S molecule or K2S variant-fusion protein and to direct secretion outside the prokaryotic host cell. Typically, the majority of the tPA, tPA variant, K2S molecule or K2S variant is secreted and can then be purified by appropriate methods such as ammonium sulfate precipitation and/or affinity chromatography and further purification steps. The invention also includes the use of inducers such as IPTG or IPTG in combination with glycerol, the improvement of the incubation condition and harvesting period to maximize the amount of active protein.

In a preferred embodiment, said DNA encoding the OmpA signal peptide may be fused to a short peptide characterized by the amino acid sequence SEGN or SEGNSD or the coding nucleic acid sequence TCTGAGGGAAAC (SEQ ID NO:20) or TCTGAGGGAAACAGTGAC (SEQ ID NO:1) and located in the N-terminal portion or at the N-terminal portion of the tPA, tPA variant, K2S molecule or K2S variant. Thus, preferably, said fusion protein comprises OmpA-SEGNSD-tPA, -tPA-variant, -K2S-molecule or -K2S-variant. Even more preferred, said amino acids characterized by SEGN or SEGNSD may be carry a point mutation or may be substituted by a non-natural amino acid. Even more preferred, there may be an amino acid or non-amino acid spacer between OmpA and SEGN or SEGNSD and the tPA, tPA variant, K2S molecule or K2S variant.

Thus, in a preferred method according to the invention said the prokaryotic cell contains and expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S

variant operably linked to the DNA coding for the signal peptide OmpA which is operably linked to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC or a functional derivative thereof.

The method according to the invention comprises prokaryotic host cells such as, but not limited to *Escherichia coli* (*E. coli*), *Bacillus subtilis*, *Streptomyces*, *Pseudomonas*, e.g. *Pseudomonas putida*, *Proteus mirabilis*, *Saccharomyces*, *Pichia* or *Staphylococcus*, e.g. *Staphylococcus carnosus*. Preferably said host cells according to the invention are Gram-negative bacteria.

Preferably, a method according to the invention is also characterised in that the prokaryotic cell is *E. coli*. Suitable strains include, but are not limited to *E. coli* XL-1 blue, BL21(DE3), JM109, DH series, TOP10 and HB101.

Preferably, a method according to the invention is also characterised in that the following steps are carried out:

- a) the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant is amplified by PCR;
- b) the PCR product is purified;
- c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked upstream to the DNA coding for the OmpA signal sequence and linked downstream to the DNA coding for gpIII of said vector;
- d) that a stop codon is inserted between said tPA, tPA variant, K2S molecule or K2S variant and gpIII;
- e) said vector is expressed by the prokaryotic cell
- f) the tPA, tPA variant, K2S molecule or K2S variant is purified.

For step a) according to the invention the choice / design of the primers is important to clone the DNA in the right location and direction of the expression vector (see example 1). Thus, the primers as exemplified in example 1 and figure 4 comprise an important aspect of the present invention. With gp III of step c) gene protein III is meant which is present mainly in phagemid vectors. The stop codon is inserted to avoid transcription of gp III thus eventually leading to secretion of the tPA, tPA variant, K2S molecule or K2S variant of interest. Any suitable method for insertion of the stop codon may be employed such as site-directed mutagenesis (e.g. Weiner MP, Costa GL (1994) PCR Methods Appl 4(3):S131-136; Weiner MP, Costa GL, Schoettlin W, Cline J, Mathur E, Bauer JC (1994) Gene 151(1-2):119-123; see also example 1).

Any vector may be used in the method according to the invention, preferably said vector is a phagemid vector (see below).

Preferably, a method according to the invention is also characterised in that the tPA, tPA variant, K2S molecule or K2S variant is selected from human tissue plasminogen activator (tPA, figure 16) or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof. Such fragments, allelic variants, functional variants, variants based on the degenerative nucleic acid code, fusion proteins with an tPA protein according to the invention, chemical derivatives or a glycosylation variant of the tPA proteins according to the invention may include one, several or all of the following domains or subunits or variants thereof:

1. Finger domain (4-50)
2. Growth factor domain (50-87)
3. Kringle 1 domain (87-176)
4. Kringle 2 domain (176-262)
5. Protease domain (276-527)

The numbering/naming of the domains is according to Genbank accession number GI 137119 or Nature 301 (5897), 214-221 (1983).

More preferably, a method according to the invention is also characterised in that the tPA, tPA variant, K2S molecule or K2S variant is selected from the Kringle 2 (4.) plus Serine protease (5.) K2S variant of human tissue plasminogen activator or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

More preferably, a method according to the invention is also characterised in that the vector is a phagemid vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII.

More preferably, a method according to the invention is also characterised in that the vector is the pComb3HSS phagemid (see also example 1).

More preferably, a method according to the invention is also characterised in that the DNA sequence comprises or consists of the following DNA sequence encoding OmpA and K2S or a functional variant thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCG
TGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGAT
CCTGATAGGCAAGGTTTACACAGCACAGAACCCCAAGTGCCCAGGCACTGGGCCTGG

GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG
 CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC
 GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA
 CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGG
 AGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC
 CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC
 ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG
 TCCATAAGGAATTTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA
 AATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC
 CCCCCGCGGACCTGCAGCTGCCGACTGGACGGAGTGTGAGCTCTCCGGCTACGGC
 AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTGAGA
 CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC
 AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGA
 CGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA
 CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT
 GTGTACACAAAGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCG
 (SEQ ID NO:2)

More preferably, a method according to the invention is also characterised in that the DNA
 Sequence of OmpA comprises or consists of the following sequence or a functional variant
 thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
 CAGGCGGCC (SEQ ID NO:3).

Said DNA encodes the following amino acid sequence of OmpA. OmpA thus comprises or
 consists of a protein characterized by the following amino acid sequence or a fragment, a
 functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant
 thereof as part of the invention:

MKKTALIAVALAGFATVAQAA (SEQ ID NO:21).

The untranslated region may contain a regulatory element, such as e.g. a transcription initiation
 unit (promoter) or enhancer. Said promoter may, for example, be a constitutive, inducible or
 development-controlled promoter. Preferably, without ruling out other known promoters, the
 constitutive promoters of the human Cytomegalovirus (CMV) and Rous sarcoma virus (RSV),
 as well as the Simian virus 40 (SV40) and Herpes simplex promoter. Inducible promoters
 according to the invention comprise antibiotic-resistant promoters, heat-shock promoters,
 hormone-inducible „Mammary tumour virus promoter“ and the metallothioneine promoter.

Preferred promoters include T3 promoter, T7 promoter, Lac/ara1 and Ltet0-1.

More preferably, a method according to the invention is also characterised in that the DNA of the
 tPA, tPA variant, K2S molecule or K2S variant is preceded by a lac promoter and/or a
 ribosomal binding site such as the Shine-Dalgarno sequence (see also example).

More preferably, a method according to the invention is also characterised in that the DNA coding for the tPA, tPA variant, K2S molecule or K2S variant is selected from the group of DNA molecules coding for at least 90% of the amino acids 87 – 527, 174 – 527, 180 – 527 or 220 – 527 of the human tissue plasminogen activator protein.

More preferably, a method according to the invention is also characterised in that the DNA Sequence of K2S comprises or consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCAGCA
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG
CAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATA
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTT
CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACCGGGT
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT
CGTCCCGCTGTGCCCAGGAGAGCAGCGTGCTCCGCACTGTGTGCCTTCCCCCGGCGG
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG
GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACTTGCACGACGCCTGCCA
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG
CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA
AGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCGTGA (SEQ ID NO:4).

The present invention also relates to variants of the before-mentioned nucleic acid molecules due to the degenerate code or to fragments thereof, nucleic acids which hybridize to said nucleic acids under stringent conditions, allelic or functional variants. The invention also relates to nucleic acids comprising said K2S nucleic acid fused to the nucleic acid encoding another protein molecule.

Stringent conditions as understood by the skilled person are conditions which select for more than 85 %, preferred more than 90 % homology (Sambrook et al. 1989; Molecular Cloning: A

Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York). The hybridisation will be carried out e.g. in 6x SSC/ 5x Denhardt's solution/ 0,1 % SDS (SDS: sodium dodecylsulfate) at 65 °C. The degree of stringency is decided in the washing step. Thus, for example for a selection of DNA-sequences with approx. 85 % or more homology, the conditions 0,2 x SSC/ 0,01 % SDS/ 65 °C and for a selection of DNA-sequences of approx. 90 % or more homology the conditions 0,1x SSC/ 0,01 % SDS/ 65 °C are suitable. The composition of said reagents is described in Sambrook et al. (1989, supra).

Another important part of the present invention is a variant of human tissue plasminogen activator comprising of or consisting of the Kringle 2 (4.) plus Serine protease (5.) (abbreviated K2S) protein or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

The numbering/naming of the domains is according to Genbank accession number GI 137119 or Nature 301 (5897), 214-221 (1983), wherein the Kringle 2 domain extends from amino acid 176-262 and the protease domain from 276-527. Thus, according to the invention, a preferred K2S molecule may include amino acids 176-527 including the amino acids between Kringle 2 and the protease (amino acids 263 to 275; exemplified in fig. (structure A)). A K2S molecule according to the invention comprises the minimal part of the Kringle 2 domain and the protease domain still retaining protease activity and fibrin binding activity (measured as exemplified in the description/example). Said K2S molecule according to the invention comprises the amino acids SEGN or SEGNSD in its N-terminal portion (see infra). A preferred K2S molecule does not include amino acids 1 to 3 or 1 to 5 of the tPA molecule. Preferably, a K2S molecule according to the invention has the amino acid Asn at positions 177 and 184, i.e. it does not require the modifications as disclosed in Waldenström for improved producibility with a method according to the invention. Thus, a preferred K2S molecule according to the invention has the native amino acid sequence (no mutation) as opposed to the molecules known from the prior art. Most preferred, said K2S molecule according to the invention is a molecule characterized by the native amino acid sequence or parts thereof, does neither have amino acids 1 to 3 nor 1 to 5 of tPA and comprises N-terminally the amino acids SEGN or SEGNSD for improved producibility and/or correct folding of the molecule.

It is essential that the K2S protein according to the invention comprises in its N-terminal portion a peptide characterized by the amino acid sequence SEGN which advantageously allows commercial production with a method as described supra leading to a correctly folded, secreted

K2S protein. Said 4 amino acids characterized by SEGN may have one or several amino acids more N-terminal, however said amino acids have to be located in the N-terminal portion as opposed to the C-terminal portion. Most preferably, said amino acids are located at the N-terminal portion. Preferably, the amino acids characterized by SEGN may carry a point mutation or may be substituted by a non-natural amino acid.

Thus, in another important embodiment the invention relates to a K2S protein characterized in that it comprises the amino acids defined by the sequence SEGN or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

Such fragments are exemplified e.g. in figure 10 (Structure B-1) and figure 11 (Structure B-2) extending from amino acids 193-527. Structure B-1 has the native amino acid Cys in position 261, wherein in B-2 the amino acid is substituted by Ser. Further fragments according to the invention comprising the amino acids 220-527 (fig. 14, structure C) or comprising the amino acids 260-527 (fig. 15, structure D) may be modified according to the invention by addition of the amino acids SEGN and/or substitution of Cys-261 by Ser. The artisan can determine the minimal length of a K2S molecule according to the invention in order to retain its biological function and generate a K2S molecule with improved producibility and/or correct folding by adding the amino acids SEGN in the N-terminal portion. Thus, another preferred embodiment is said minimal K2S molecule with SEGN at its N-terminal portion.

In another important embodiment the invention relates to a K2S protein characterized in that it comprises the amino acids defined by the sequence SEGNSD or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof. Such fragments are exemplified e.g. in figure 12 (Structure B-3) and figure 13 (Structure B-4) extending from amino acids 191-527. Structure B-3 has the native amino acid Cys in position 261, wherein in B-4 the amino acid is substituted by Ser. Further fragments according to the invention comprising the amino acids 220-527 (fig. 14, structure C) or comprising the amino acids 260-527 (fig. 15, structure D) may be modified according to the invention by addition of the amino acids SEGNSD and/or substitution of Cys-261 by Ser. The artisan can determine the minimal length of a K2S molecule according to the invention in order to retain its biological function and generate a K2S molecule with improved producibility and/or correct folding by adding the amino acids SEGNSD in the N-terminal portion. Thus, another preferred embodiment is said minimal K2S molecule with SEGNSD at its N-terminal portion.

Another more preferred embodiment of the present invention relates to a K2S protein comprising a protein characterized by the following amino acid sequence or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant thereof:

5 SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQUALGLGKHNY
CRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW
QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTIVLGRTYRVVPGEEEQ
KFEVEKYIVHKEFDDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC
ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA
10 NLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNM
RP* (SEQ ID NO:11).

According to the invention, * means STOP (i.e. encoded by a stop codon). This K2S molecule is exemplified in figure 8.

One variant of the K2S molecule according to the invention relates to a fusion protein of K2S
15 being fused to another protein molecule.

Another more preferred embodiment of the present invention relates to a K2S protein consisting of a protein characterized by the following amino acid sequence:

20 SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQUALGLGKHNY
CRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW
QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTIVLGRTYRVVPGEEEQ
KFEVEKYIVHKEFDDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC
ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA
NLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNM
RP* (SEQ ID NO:11).

25 Said K2S molecules may be encoded by a DNA molecule as described supra.

Another important aspect of the invention relates to a DNA molecule characterized in that it is coding for:

- a) the OmpA protein or a functional derivative thereof operably linked to
- b) a DNA molecule coding for a polypeptide containing the kringle 2 domain and the serine
30 protease domain of tissue plasminogen activator protein.

More preferably, a DNA molecule according to the invention is also characterised in that the DNA sequence comprises or consists of the following DNA sequence encoding OmpA and K2S or a functional variant thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCG
TGGCACGCACAGCCTCACCAGATCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGAT
CCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGG
GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG
CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC
GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGG
AGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC
CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC
ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG
TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA
AATCGGATTCGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC
CCCCGGCGGACCTGCAGCTGCCGGAAGTGTGAGCTCTCCGGCTACGGC
AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGA
CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC
AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTGCACGA
CGCCTGCCAGGGCGATTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA
CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT
GTGTACACAAAGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG
(SEQ ID No:2)

Said DNA molecule encodes the following fusion protein of OmpA and K2S. Said fusion protein of OmpA and K2S characterised in that it comprises or consists of a protein characterized by the following amino acid sequence or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant thereof forms an important part of the present invention:

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILI
GKVYTAQNPSAQALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLR
QYSQPQFRKGGFLADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERF
PPHHLTIVLGRITYRVVPGEEEKFEVEKYIVHKEFDDDTYDNDIALQLKSDSSRCAQES
SVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLL
NRTVTDNMLCAGDTRSGGPQANLHDACQGDGGPLVCLNDGRMTLVGIISWGLGCGQ
KDVPGVYTKVTNYLDWIRDNMRPG (SEQ ID NO:8)

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 87 -

527 of the human tissue plasminogen activator protein (numbering used herein as GI 137119 or Nature 301 (5897), 214-221 (1983).

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 174 – 527 of the human tissue plasminogen activator protein.

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 180 – 527 of the human tissue plasminogen activator protein.

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 220 – 527 of the human tissue plasminogen activator protein.

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence a) is hybridizing under stringent conditions to the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCC (SEQ ID NO:3).

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence a) consists of the following sequence:
ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCC (SEQ ID NO:3).

Another preferred aspect of the invention relates to a DNA molecule according to the invention, characterized in that said DNA sequence b) is hybridizing under stringent conditions to the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG
CAAGGTTTACACAGCACAGAACCCCAAGTGGCCAGGCACTGGGCCTGGGCAAACATA
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCCGAGAGCGGTT
CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACCGGGT

GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT
CGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG
ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG
5 GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCA
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG
CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA
10 AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO:4).

Another preferred aspect of the invention relates to a DNA molecule according to the invention,
characterized in that said DNA sequence b) consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCAGCA
CAGCCTACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG
15 CAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATA
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCCGAGAGCGGTT
20 CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATAACCGGT
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT
CGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG
25 ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG
GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGACGACGCCTGCCA
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG
30 CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA
AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO:4).

Another preferred embodiment of the invention relates to a vector containing a DNA sequence according to the invention.

Another preferred embodiment of the invention relates to a vector according to the invention, wherein said DNA sequence is preceded by a lac promoter and a ribosomal binding site. Suitable vectors according to the invention include, but are not limited to viral vectors such as e.g. Vaccinia, Semliki-Forest-Virus and Adenovirus, phagemid vectors and the like. Preferred are vectors which can be advantageously used in *E. coli*, but also in any other prokaryotic host such as pPROTet.E, pPROLar.A, members of the pBAD family, pSE family, pQE family and pCAL.

Another preferred embodiment of the invention relates to the vector pComb3HSS containing a DNA according to the invention, wherein the expression of the gp III protein is suppressed or inhibited by deleting the DNA molecule encoding said gp III protein or by a stop codon between the gene coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein and the protein III gene.

Another important aspect of the present invention relates to a prokaryotic host cell comprising a DNA molecule according to the invention.

Another important aspect of the present invention relates to a prokaryotic host cell comprising a vector according to the invention.

Another important aspect of the present invention relates to an *E. coli* host cell comprising a DNA molecule according to the invention.

Another important aspect of the present invention relates to a *E. coli* host cell comprising a vector according to the invention.

Yet another important aspect of the present invention is the use of a DNA molecule according to the invention or of a vector according to the invention or a host cell according to the invention in a method for the production of a polypeptide having the activity of tissue plasminogen activator.

Yet another important aspect of the present invention is the use according to the invention as described above, wherein said method is a method according to the invention.

Another very important aspect is a pharmaceutical composition comprising a substance obtainable by a method according to the invention and pharmaceutically acceptable excipients and carriers. An example for said substance is the K2S molecule described supra. The term "pharmaceutically acceptable carrier" as used herein refers to conventional pharmaceutical excipients or additives used in the pharmaceutical manufacturing art. Such physiologically acceptable compounds include, for example, carbohydrates, such as glucose, sucrose or dextrans,

antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients (see also e.g. Remington's Pharmaceutical Sciences (1990, 18th ed. Mack Publ., Easton.)). Said pharmaceutical composition according to the invention can be advantageously administered intravenously as a bolus, e.g. as a single bolus for 5 to 10 seconds intravenously.

The invention further relates to the use of substances obtainable by a method according to the invention in the manufacture of a medicament in the treatment of stroke, cardiac infarction, acute myocardial infarction, pulmonary embolism, any artery occlusion such as coronary artery occlusion, intracranial artery occlusion (e.g. arteries supplying the brain), peripherally occluded arteries, deep vein thrombosis or related diseases associated with unwanted blood clotting.

The following example is intended to aid the understanding of the invention and should in no way be regarded as limiting the scope of the invention.

Example 1

MATERIALS AND METHODS

Primer design. In order to amplify a specific part of tPA gene, a pair of primers SK2/174 [5' GAGGAGGAGGTGGCCCAGGCGGCCTCTGAGGGAAACAGTGAC 3'] (SEQ ID NO:22) and ASSP

[5' GAGGAGGAGCTGGCCGGCCTGGCCCGGTCGCATGTTGTCACG 3'] (SEQ ID NO:23) were synthesized (Life Technologies, Grand Island, NY). These primers were designed based on the human tPA gene retrieved from NCBI databases (g137119). They were synthesized with Sfi I end cloning sites (underlined) in such a way that the reading frame from the ATG of the gpIII gene in phagemid vector, pComb3HSS, will be maintained throughout the inserted sequence.

Another primer set for site-directed mutagenesis was designed to anneal at the sequence situated between K2S gene and gene III in pComb3H-K2S. The sequence of primers with mutation bases (underlined) for generating a new stop codon were MSTPA

[5' ACATGCGACCGTGACAGGCCGGCCAG 3'] (SEQ ID NO:24) and MASTPA

[5' CTGGCCGGCCTGTCACGGTTCGCATGT 3'] (SEQ ID NO:25).

Amplification of K2S gene by PCR. One µg SK2/174 and ASSP primers together with 50 ng of p51-3 template (obtained from Dr. Hiroshi Sasaki, Fujisawa Pharmaceutical, Japan) were suspended in 100 µl PCR mixture. An amount of 2.5 U Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN) was finally added to the solution. The titrated amplification condition was initiated with jump start at 85°C for 4 min, then denaturation at 95°C for 50 sec, annealing at 42°C for 50 sec, extension at 72°C for 1.5 min. Thirty five rounds were repeatedly performed. The mixture was further incubated at 72°C for 10 min. The amplified product of 1110 bp was subsequently purified by QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). The correctness of purified product was confirmed by restriction enzymes.

Construction of phagemid expressing K2S. The purified PCR product of K2S and pComb3HSS phagemid (kindly provided by Dr. Carlos F. Barbas, Scripps Institute, USA) were digested with Sfi I (Roche Molecular Biochemicals, Indianapolis, IN) to prepare specific cohesive cloning sites. Four µg of the purified PCR product was digested with 60 U of Sfi I at 50°C for 18 h. For pComb3HSS, 20 µg of phagemid vectors were treated with 100 U of Sfi I. Digested products of purified PCR product of K2S and pComb3HSS (~3300 bp) were subsequently gel-purified by the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). T4 ligase (Roche Molecular Biochemicals, Indianapolis, IN) of 5 U were introduced to the mixture of 0.7 µg of purified Sfi I-digested pComb3HSS and 0.9 µg of purified Sfi I-digested PCR product. Ligation reaction was incubated at 30°C for 18 h. The newly constructed phagemid was named pComb3H-K2S.

Transformation of E. coli XL-1 Blue. Two hundred µl of CaCl₂ competent E. coli XL-1 Blue (Stratagene, La Jolla, CA) were transformed with 70 ng of ligated or mutated product. The transformed cells were propagated by spreading on LB agar containing 100 µg/ml ampicillin and 10 µg/ml tetracycline (Sigma, Saint Louis, MO). After cultivation at 37°C for 18 h several antibiotic resistant colonies were selected for plasmid minipreps by using the alkaline lysis method. Each purified plasmid was subjected to Sfi I restriction site analysis. A transformant harboring plasmid with the correct Sfi I restriction site(s) was subsequently propagated for 18 h at 37°C in 100 ml LB broth with ampicillin 100 µg/ml and tetracycline 10 µg/ml. A plasmid maxiprep was performed using the QIAGEN Plasmid Maxi Kit (QIAGEN, Hilden, Germany). The purified plasmid was reexamined for specific restriction sites by Sfi I and sequenced by

AmpliTaQ DNA Polymerase Terminator Cycle Sequencing Kit (The Perkin-Elmer Corporation, Forster City, CA).

Site-directed mutagenesis of pComb3H-K2S. 10 ng of pComb3H-K2S template were mixed with 125 ng of MSTPA and MASTPA primers. PfuTurbo DNA polymerase (Stratagene, LA Jolla, CA) of 2.5 U was added to the mixture for cycle amplification. The reaction started with one round of 95°C for 30 sec. Then it was followed by 16 rounds consisting of 95°C for 30 sec, 55°C for 1 min, and 68°C for 9 min. The reaction tube was subsequently placed on ice for 2 min. In order to destroy the template strands, 10 U of Dpn I restriction enzyme (Stratagene, LA Jolla, CA) were added to the amplification reaction and incubated for 1 h at 37°C. This synthesized product (MpComb3H-K2S) was further used to transform E. coli XL-1 Blue.

Preparation of phage-display recombinant-K2S. After pComb3H-K2S was transformed to XL-1 Blue, the phage display technique was performed. A clone of pComb3H-K2S transformed E. coli XL-1 Blue was propagated in 10 ml super broth containing ampicillin 100 µg/ml and tetracycline 10 µg/ml at 37°C until the O.D. [600 nm] of 1.5 was reached. The bacterial culture was subsequently propagated in 100 ml of the same medium and culture for 2 h. An amount of 10^{12} pfu of VCSM13 helper phage (Stratagene, La Jolla, CA) was used to infect the transformed E. coli XL-1 Blue. After 3 h incubation, kanamycin at a final concentration of 70 µg/ml final concentration was added to culture. The culture was left shaking (200 RPM) for 18 h at 37°C. Bacteriophages which harbored K2S on gp3 (K2S-φ) were then harvested by adding 4% w/v PEG MW 8000 (Sigma, Saint Louis, MO) and 3% w/v NaCl. Finally, the harvested phage was resuspended in 2 ml PBS pH 7.4. The phage number was determined by infecting E. coli XL-1 Blue. The colony-forming unit per milliliter (cfu/ml) was calculated as described previously (21).

Expression of recombinant-K2S in shaker flasks. MpComb3H-K2S transformed E. coli XL-1 Blue was cultivated in 100 ml super broth (3% w/v tryptone, 2% w/v yeast extract and 1% w/v MOPS) at pH 7.0 in the presence of ampicillin (100 µg/ml) at 37°C until an O.D. [600 nm] of 0.8 was reached. Subsequently, the protein synthesis was induced by 1 mM of IPTG (Promega, Madison, WI). The bacteria were further cultured shaking (200 RPM) for 6 h at 30°C. The culture supernatant was collected and precipitated with 55% saturated ammonium sulfate (32).

The precipitate was reconstituted with PBS, pH 7.2, and dialysed in the same buffer solution at 4°C for 18 h. Periplasmic proteins from bacterial cells were extracted by using a chloroform shock as previously described by Ames et al. (2).

5 Immunoassay quantification of recombinant-K2S. In order to detect r-K2S, solid phase was coated with monoclonal anti-kringle 2 domain (16/B) (generously provided by Dr. Ute Zacharias, Central Institute of Molecular Biology, Berlin-Buch, Germany). The standard ELISA washing and blocking processes were preformed. Fifty µl of 10^{11} cfu/ml of K2S-φ or secretory r-K2S were added into each anti-kringle 2 coated well. Antigen-antibody detection was carried out
10 as follows. Either sheep anti-M13 conjugated HRP (Pharmacia Biotech, Uppsala, Sweden) or sheep anti-tPA conjugated HRP (Cedarlane, Ontario, Canada), was added to each reaction well after the washing step. The substrate TMB was subjected to every well and the reaction was finally ceased with H₂SO₄ solution after 30 min incubation. The standard melanoma tPA 86/670 (National Institute for Biological Standards and Control, Hertfordshire, UK) was used as
15 positive control.

Amidolytic activity assay. A test kit for the detection of tPA amidolytic activity was purchased from Chromogenix (Molndal, Sweden). The substrate mixture containing plasminogen and S-2251 was used to determine serine protease enzymatic activity. The dilution of 10^{-2} of each
20 ammonium precipitated sample was assayed with and without stimulator, human fibrinogen fragments. The assay procedure was according to the COASET t-PA manual.

SDS-PAGE and immunoblotting. The dialysed precipitate-product from culture supernatant was further concentrated 10 folds with centricon 10 (AMICON, Beverly, MA). The concentrated
25 sample was subjected to protein separation by SDS-PAGE, 15% resolving gel, in the reducing buffer followed by electroblotting to nitrocellulose. The nitrocellulose was then blocked with 4% skimmed milk for 2 hr. In order to detect r-K2S, a proper dilution of sheep anti-tPA conjugated HRP was applied to the nitrocellulose. The immunoreactive band was visualized by a sensitive detection system, Amplified Opti-4CN kit (BIORAD, Hercules, CA).

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Copolymerized plasminogen polyacrylamide gel electrophoresis. An 11% resolving polyacrylamide gel was copolymerized with plasminogen and gelatin as previously described by

Heussen et al. (14). The stacking gel was prepared as 4 % concentration without plasminogen and gelatin. Electrophoresis was performed at 4°C at a constant current of 8 mA. The residual SDS in gel slab was removed after gentle shaking at room temperature for 1h in 2.5% Triton X-100. Then the gel slab was incubated in 0.1 M glycine-NaOH, pH 8.3, for 5 h at 37°C. Finally, the gel slab was stained and destained by standard Coomassie brilliant blue (R-250) dying system. The location of the peptide harboring enzymatic activity was not stained by dye in contrast to blue-paint background.

RESULTS

Construction of K2S gene carrying vector. From the vector p51-3 we amplified the kringle 2 plus the serine protease portion of tPA (Ser¹⁷⁴ in kringle 2 domain to Pro⁵²⁷ in the serine protease) using primers SK2/174 and ASSP. The amplified 1110 bp product was demonstrated by agarose gel electrophoresis (Fig. 1, lane 2) and was inserted into pComb3HSS phagemid by double Sfi I cleavage sites on 5' and 3' ends in the correct reading frame. Thus a new vector, pComb3H-K2S, harboring the K2S was generated. In this vector K2S is flanked upstream by the OmpA signal sequence and downstream by gp3. The correct insertion of K2S was verified both by restriction analysis with Sfi I (Fig. 2, lane 3), PCR-analysis (demonstration of a single band at 1110 bp), and DNA sequencing. The schematic diagram of pComb3H-K2S map is given in Fig. 3.

Phage-displayed r-K2S. VCSM13 filamentous phage was used to infect pComb3H-K2S transformed E. coli XL-1 Blue, X[K2S]. VCSM13 was propagated and incorporated the K2S-gp3 fusion protein during the viral packaging processes. The harvested recombinant phage (K2S- ϕ) gave a concentration of 5.4×10^{11} cfu/ml determined by reinfecting E. coli XL-1 Blue with PEG-precipitated phages. These recombinant phage particles were verified for the expression of r-K2S by sandwich ELISA. The phage-bound heterologous K2S protein was recognized by the monoclonal anti-kringle 2 antibody (16/B) by using sheep anti-tPA conjugated HRP antibody detection system. The absorbance of this assay was 1.12 ± 0.03 (Table 1). The amount of K2S detectable on 10^{12} phage particles is equal to 336 ng of protein in relation to the standard melanoma tPA. In order to corroborate that K2S-gp3 fusion protein was associated with phage particles, sheep anti-tPA conjugated HRP antibody was substituted by sheep anti-M13 antibody conjugated HRP. This immuno-reaction exhibited an absorbance of 1.89 ± 0.07 (Table 1). In

contrast, if the capture antibody was sheep anti-M13 antibody, extremely low K2S was observed with sheep anti-tPA antibody conjugated HRP; the absorbance was only 0.17 ± 0.01 (Table 1). This suggested that only a minority of purified phage particles carried K2S-gp3 fusion protein. VCSM13 prepared from non-transformed XL-1 Blue was used as a negative control.

Construction of MpComb3H-K2S. We generated a stop codon between K2S and gp3 in pComb3H-K2S with the aid of the mutagenic primers (MSTPA and MASTPA) (Fig. 4). In order to enrich the newly synthesized and mutated MpComb3H-K2S, the cycle amplification mixture was thoroughly digested with Dpn I to degrade the old dam methylated pComb3H-K2S template (Dpn I prefers dam methylated DNA). After transforming of *E. coli* XL-1 Blue with MpComb3H-K2S, a transformant XM[K2S] was selected for further study. As a consequence of bp substitution, one Sfi I cleavage site close to the 3' end of K2S gene was lost after site-directed mutagenesis. A linear version of Sfi I cleaved MpComb3H-K2S was observed at 4319 bp without the appearance of inserted K2S gene fragment (Fig. 5, lane 3). Thus, the K2S gene encoding by MpComb3H-K2S was expressed in non-gp3 fusion form in XM[K2S].

Expression and purification of K2S. K2S expression in XM[K2S] was induced by IPTG. r-K2S was detectable by using ELISA both in the periplasmic space and in the culture supernatant. The amount of the heterologous protein in each preparation was determined by sandwich ELISA and related to the standard tPA. From 100 ml of the bacterial culture in shaker flask with the O.D. [600 nm] of 50, the periplasmic fraction yielded 1.38 μ g of r-K2S (approximately 32%) whereas 2.96 μ g of r-K2S (approximately 68%) was obtained in the ammonium precipitated culture supernatant. Sandwich ELISA was used to verify the PEG precipitated phage from VCSM13 infected XM[K2S]. No r-K2S captured by monoclonal anti-kringle 2 antibody was detected by anti-M13 conjugated HRP, indicating that K2S is not presented on the phage particles if gp3 is missing.

Amidolytic activity measurement. If serine protease domain is present in the sample, plasminogen will be converted to plasmin. The produced plasmin will further digest the S-2251 substrate to a colour product, p-nitroaniline, which has a maximum absorbance at 405 nm. The specific activity of the recombinant product is in accordance with the absorbance. The fibrinogen-dependent enzymatic activity of each sample i.e. K2S- ϕ , periplasmic r-K2S or culture

supernatant r-K2S, was evaluated and compared. Both K2S- ϕ and periplasmic r-K2S illustrated notably low enzymatic activity, which was below the sensitivity of the test (0.25 IU/ml). The culture supernatant r-K2S gave the fibrinogen-dependent enzymatic activity of 7 IU/ml. Thus, from 100 ml culture we obtained a total of 700 IU enzymatic activity. Without fibrinogen no enzymatic activity of the r-K2S purified from culture supernatant was observed - whereas standard melanoma tPA showed some activity.

Demonstration of recombinant protein by immunoblotting. Partially purified K2S from culture supernatant of XM[K2S] revealed a molecular mass of 39 kDa by using sheep anti-tPA antibodies (Fig. 6). The negative control, partially purified culture supernatant of non-transformed XL1-Blue, contained no reactive band with a similar size.

Localization of active enzyme by PAGE. The plasminogen has been copolymerized and immobilized with gelatin in the polyacrylamide gel prior to electrophoresis. The ammonium sulfate precipitated culture supernatants of E. coli XL-1 Blue, E. coli XL-1 Blue transformed with pComb3HSS and XM[K2S] were analyzed (Fig. 7). All samples were processed in non-reducing condition to preserve the correct conformation and activity of the serine protease domain. Transparent areas of serine protease digested plasminogen were observed only in the ammonium sulfate precipitated culture supernatants of XM[K2S] at 34 and 37 kDa positions. The other samples gave no clearing zones. The positive control lane of standard melanoma tPA also demonstrated enzymatic activity at 66 and 72 kDa positions.

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FIGURE LEGENDS

FIG. 1. Validation of PCR amplification product of the K2S gene from the p51-3 vector by using SK2/174 and ASSP primers. Lane 1 shows 1 kb marker (Roche Molecular Biochemicals, Indianapolis, IN). Lane 2 was loaded with 1 µl of amplified product. A single band at 1110 bp is depicted. The electrophoresis was performed on a 1% agarose gel.

FIG. 2. Identification of inserted K2S gene at 1110 bp (*) after Sfi I digested pComb3H-K2S was demonstrated in lane 3. Lane 1 shows 1 kb marker. Lane 2 was loaded with uncut pComb3H-K2S. The electrophoresis was performed on a 1% agarose gel.

FIG. 3. Scheme of pComb3H-K2S showing two Sfi I cloning sites into which the K2S gene was inserted. Signal sequence (OmpA), ribosome binding site (RIBS), lac promotor, and gpIII gene are also depicted.

FIG. 4. Schematic diagram of the mutation site at the junction between the K2S and gpIII genes on pComb3H-K2S. The annealing site of pComb3H-K2S is bound with a set of mutation primers (MSTPA and MASTPA) containing modified oligonucleosides (underlined). After performing the cycle amplification, the Sfi I site 1 (in bold) is modified and lost in the newly synthesized strand.

FIG. 5. Characterization of newly synthesized MpComb3H-K2S by the Sfi I restriction enzyme. A single band at 4319 bp that refers to a single cleavage site of MpComb3H-K2S is observed in lane 3. No inserted K2S band at 1110 bp can be visualized. Lane 1 shows 1 kb marker. Lane 2 was loaded with uncut MpComb3H-K2S. The electrophoresis was performed on a 1% agarose gel.

FIG. 6. Identification of immunological reactive band with of recombinant DNA-derived protein purified from XM[K2S] culture supernatant with sheep anti-tPA conjugated HRP. Lane 1 was loaded with 40 ng of standard melanoma tPA (86/670); which showed the reactive band at 70 kDa. The partially purified and concentrated culture supernatants from non-transformed E. coli

XL1- Blue and XM[K2S] were applied to lane 2 and 3 respectively. The distinct reactive band was particularly demonstrated in lane 3 at 39 kDa.

FIG. 7. Molecular weight determination of extracellular r-K2S harboring active serine protease domain by copolymerized plasminogen polyacrylamide gel electrophoresis. Lane 1 contained the indicated molecular weight standards ($\times 10^3$), SDS-6H (Sigma, Saint Louis, MO). Fifty μ g of the 55% saturated ammonium sulfate precipitated culture supernatant of XL-1 Blue, XI-1 Blue transformed with pComb3HSS, and XM[K2S] were loaded in lane 2, 3, and 4 respectively. Lane 5 contained 50 mIU of standard melanoma tPA (86/670). Transparent zones of digested plasminogen in polyacrylamide gel are visible only in lane 4 at molecular weight of 34 and 37 kDa (B) and lane 5 at molecular weight of 66 and 72 kDa (A).

FIG. 8. Structure A (SEQ ID NO:11)

Native K2S molecule from amino acids 174-527 without modification.

FIG. 9. Structure B-0 (SEQ ID NO:12)

Native K2S molecule from amino acids 197-527 without modification.

FIG. 10. Structure B-1 (SEQ ID NO:13)

K2S molecule from amino acids 193-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGN were added at the N-terminal portion.

FIG. 11. Structure B-2 (SEQ ID NO:14)

K2S molecule from amino acids 193-527, as in Fig. 10, wherein Cys-261 was exchanged for Ser.

FIG. 12. Structure B-3 (SEQ ID NO:15)

K2S molecule from amino acids 191-527, wherein to Structure B-0 of Fig. 9 the amino acids SEGNSD were added at the N-terminal portion.

FIG. 13. Structure B-4 (SEQ ID NO:16)

K2S molecule from amino acids 191-527, as in Fig. 12, wherein Cys-261 was exchanged for Ser.

FIG. 14. Structure C (SEQ ID NO:17)

Native K2S molecule from amino acids 220-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13.

FIG. 15. Structure D (SEQ ID NO:18)

Native K2S molecule from amino acids 260-527 without modification. This molecule may be further modified in a similar manner as disclosed for structure B in figures 10-13.

FIG. 16. tPA molecule (SEQ ID NO:19)

TABLE 1. Detection of r-K2S molecule in phage preparation by sandwich ELISA

Capture antibody	Tracer antibody (conjugated HRP)			
	Anti-tPA		Anti-M13	
	K2S- ϕ	VCSM13 ^a	K2S- ϕ	VCSM13
Anti-kringle 2 ^b	1.12 \pm 0.04 ^c	0.12 \pm 0.03	1.89 \pm 0.02	0.16 \pm 0.02
Anti-M13	0.17 \pm 0.01	0.14 \pm 0.05	1.91 \pm 0.02	1.88 \pm 0.03

^a VCSM13 was harvested from XL-1 Blue transformed with pComb3HSS.

^b Mouse monoclonal anti-kringle 2 (16/B) was used. The other antibodies were prepared from sheep immunoglobulin.

^c Value is mean of absorbance of each sample which was assayed in triplicate.

Claims

1. Method for the production of recombinant DNA-derived tissue plasminogen activator (tPA),
5 a tPA variant, a Kringle 2 Serine protease molecule (K2S) or a K2S variant in prokaryotic cells,
wherein said tPA, tPA variant, K2S molecule or K2S variant is secreted extracellularly as an
active and correctly folded protein, characterized in that the prokaryotic cell contains and
expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S
variant operably linked to the DNA coding for the signal peptide OmpA or a functional
10 derivative thereof.
2. Method according to claim 1, characterised in that said the prokaryotic cell contains and
expresses a vector comprising the DNA coding for said tPA, tPA variant, K2S molecule or K2S
variant operably linked to the DNA coding for the signal peptide OmpA which is operably linked
to the nucleic acid molecule defined by the sequence TCTGAGGGAAACAGTGAC (SEQ ID
15 NO:1) or a functional derivative thereof.
3. Method according to claim 1 or 2, characterised in that the prokaryotic cell is *E. coli*.
4. Method according to one of claims 1 to 3, characterised in that the the following steps are
carried out:
 - a) the DNA encoding the tPA, tPA variant, K2S molecule or K2S variant is amplified by PCR;
 - 20 b) the PCR product is purified;
 - c) said PCR product is inserted into a vector comprising the DNA coding for OmpA signal
peptide and the DNA coding for gpIII in such a way that said PCR product is operably linked
upstream to the DNA coding for the OmpA signal sequence and linked downstream to the DNA
coding for gpIII of said vector;
 - 25 d) that a stop codon is inserted between said tPA, tPA variant, K2S molecule or K2S variant and
gpIII;
 - e) said vector is expressed by the prokaryotic cell;
 - f) the tPA, tPA variant, K2S molecule or K2S variant is purified.
5. Method according to one of claims 1 to 4, characterised in that the vector is a phagemid
30 vector comprising the DNA coding for OmpA signal peptide and the DNA coding for gpIII.
6. Method according to one of claims 1 to 5, characterised in that the vector is the pComb3HSS
phagemid.

7. Method according to one of claims 1 to 6, characterised in that the DNA Sequence of OmpA linked upstream to K2S comprises the following sequence or a functional variant thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCG
TGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGAT
CCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGG
GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG
CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC
GGCCTGAGACAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGA
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCGG
AGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC
CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAAC
ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG
TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA
AATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC
CCCCGGCGGACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGC
AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGA
CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC
AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACTTGCACGA
CGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA
CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT
GTGTACACAAAGGTTACCAACTACCTAGACTGGATTTCGTGACAACATGCGACCG
(SEQ ID NO:2)

8. Method according to one of claims 1 to 7, characterised in that the DNA Sequence of OmpA comprises the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCC (SEQ ID NO:3)

9. Method according to one of claims 1 to 8, characterised in that the DNA Sequence of OmpA consists of the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCC (SEQ ID NO:3)

10. Method according to one of claims 1 to 9, characterised in that the DNA of the tPA, tPA
variant, K2S molecule or K2S variant is preceeded by a lac promotor and/or a ribosomal binding
site.

11. Method according to one of claims 1 to 10, characterised in that the DNA coding for the tPA,
tPA variant, K2S molecule or K2S variant is selected from the group of DNA molecules coding
for at least 90% of the amino acids 87 – 527, 174 – 527, 180 – 527 or 220 – 527 of the human
tissue plasminogen activator protein.

12. Method according to one of claims 5 to 11, characterised in that the DNA Sequence of K2S
comprises the following sequence or a functional variant thereof or a variant due to the
degenerate nucleotide code:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTGAGCCTACCGTGGCACGCA
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGGAATTCCATGATCCTGATAGG
15 CAAGGTTTACACAGCACAGAACCCAGTGCCAGGCACTGGGCCTGGGCAAACATA
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCCGAGAGCGGTT
20 CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCCACTGCTTC
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATACCGGGT
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT
CGTCCCGCTGTGCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCCGGCGG
25 ACCTGCAGCTGCCGGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG
GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTGCACTGTACCCA
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG
TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTGCACGACGCCTGCCA
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG
30 CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA
AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO:4).

13. Method according to one of claims 5 to 12, characterised in that the DNA Sequence of K2S consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG
5 CAAGGTTTACACAGCACAGAACCCCAAGTGGCCAGGCACTGGGCCTGGGCAAACATA
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA
CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC
CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCCCCGGAGAGCGGTT
10 CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC
CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATAACCGGT
GGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAAGG
AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT
CGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCGGCGG
15 ACCTGCAGCTGCCGACTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG
GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA
TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG
TGTGCTGGAGACACTCGGAGCGGGCGGGCCCCAGGCAAACCTGCACGACGCCTGCCA
GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG
20 CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA
AGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCGTGA (SEQ ID NO:4).

14. DNA molecule characterized in that it is coding for:

- a) the OmpA protein or a functional derivative thereof operably linked to
- b) a DNA molecule coding for a polypeptide containing the kringle 2 domain and the serine
25 protease domain of tissue plasminogen activator protein.

15. DNA molecule according to claim 14, characterized in that said DNA sequence comprises the following sequence or a functional variant thereof or a variant due to the degenerate nucleotide code:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
30 CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCG
TGGCACGCACAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGAT
CCTGATAGGCAAGGTTTACACAGCACAGAACCCCAAGTGGCCAGGCACTGGGCCTGG
GCAAACATAATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG
CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC

GGCCTGAGACAGTACAGCCAGCCTCAGTTTTCGCATCAAAGGAGGGGCTCTTCGCCGA
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCCG
AGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC
CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACTGACGGTGATCTTGGGCAGAAC
5 ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG
TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA
AATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC
CCCCGGCGGACCTGCAGCTGCCGGAAGTGGACGGAGTGTGAGCTCTCCGGCTACGGC
AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGA
10 CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC
AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGA
CGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA
CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT
GTGTACACAAAGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG
15 (SEQ ID NO:5).

16. DNA molecule according to claim 14 or 15, characterized in that said DNA sequence consists of the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
20 CAGGCGGCCTCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTACGCCTACCG
TGGCACGCACAGCCTACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAAATCCATGAT
CCTGATAGGCAAGGTTTACACAGCACAGAACCCAGTGCCAGGCACTGGGCCTGG
GCAAACATAATTACTGCCGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTG
CTGAAGAACCGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGC
25 GGCCTGAGACAGTACAGCCAGCCTCAGTTTTCGCATCAAAGGAGGGGCTCTTCGCCGA
CATCGCCTCCCACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTCGCCCCG
AGAGCGGTTCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGC
CCACTGCTTCCAGGAGAGGTTTCCGCCCCACCACTGACGGTGATCTTGGGCAGAAC
ATACCGGGTGGTCCCTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTG
30 TCCATAAGGAATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGA
AATCGGATTTCGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTC
CCCCGGCGGACCTGCAGCTGCCGGAAGTGGACGGAGTGTGAGCTCTCCGGCTACGGC
AAGCATGAGGCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGA
CTGTACCCATCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGAC
35 AACATGCTGTGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTTGCACGA
CGCCTGCCAGGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGA
CTTTGGTGGGCATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGT
GTGTACACAAAGGTTACCAACTACCTAGACTGGATTCTGTGACAACATGCGACCG
(SEQ ID NO:5).

40 17. DNA molecule according to one of claims 14 to 16, characterized in that said DNA sequence
b) is coding for at least 90% of the amino acids 87 – 527 of the human tissue plasminogen
activator protein.

18. DNA molecule according to one of claims 14 to 17, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 174 – 527 of the human tissue plasminogen activator protein.

19. DNA molecule according to any one of claims 14 to 18, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 180 – 527 of the human tissue plasminogen activator protein.

20. DNA molecule according to any one of claims 14 to 19, characterized in that said DNA sequence b) is coding for at least 90% of the amino acids 220 – 527 of the human tissue plasminogen activator protein.

21. DNA molecule according to any one of claims 14 to 20, characterized in that said DNA sequence a) is hybridizing under stringent conditions to the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCC (SEQ ID NO:6).

22. DNA molecule according to any one of claims 14 to 21, characterized in that said DNA sequence a) consists of the following sequence:

ATGAAAAAGACAGCTATCGCGATTGCAGTGGCACTGGCTGGTTTCGCTACCGTGGCC
CAGGCGGCC (SEQ ID NO:6).

23. DNA molecule according to any one of claims 14 to 22, characterized in that said DNA sequence b) is hybridizing under stringent conditions to the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA
CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCCATGATCCTGATAGG
CAAGGTTTACACAGCACAGAACCCAGTGCCCAGGCACTGGGCCTGGGCAAACATA
ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC
CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA
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 5 CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA
 AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO:7).

24. DNA molecule according to any one of claims 14 to 23, characterized in that said DNA
 sequence b) consists of the following sequence:

TCTGAGGGAAACAGTGACTGCTACTTTGGGAATGGGTCAGCCTACCGTGGCACGCA
 10 CAGCCTCACCGAGTCGGGTGCCTCCTGCCTCCCGTGGAATTCATGATCCTGATAGG
 CAAGGTTTACACAGCACAGAACCCCAAGTGCCAGGCACTGGGCCTGGGCAAACATA
 ATTACTGCCGGAATCCTGATGGGGATGCCAAGCCCTGGTGCCACGTGCTGAAGAAC
 CGCAGGCTGACGTGGGAGTACTGTGATGTGCCCTCCTGCTCCACCTGCGGCCTGAGA
 CAGTACAGCCAGCCTCAGTTTCGCATCAAAGGAGGGCTCTTCGCCGACATCGCCTCC
 15 CACCCCTGGCAGGCTGCCATCTTTGCCAAGCACAGGAGGTGCGCCGGAGAGCGGTT
 CCTGTGCGGGGGCATACTCATCAGCTCCTGCTGGATTCTCTCTGCCGCCCACTGCTTC
 CAGGAGAGGTTTCCGCCCCACCACCTGACGGTGATCTTGGGCAGAACATAACCGGT
 GGTCCTTGGCGAGGAGGAGCAGAAATTTGAAGTCGAAAAATACATTGTCCATAAGG
 AATTCGATGATGACACTTACGACAATGACATTGCGCTGCTGCAGCTGAAATCGGATT
 20 CGTCCCGCTGTGCCCAGGAGAGCAGCGTGGTCCGCACTGTGTGCCTTCCCCGGCGG
 ACCTGCAGCTGCCGGAAGTGGACGGAGTGTGAGCTCTCCGGCTACGGCAAGCATGAG
 GCCTTGTCTCCTTTCTATTTCGGAGCGGCTGAAGGAGGCTCATGTCAGACTGTACCCA
 TCCAGCCGCTGCACATCACAACATTTACTTAACAGAACAGTCACCGACAACATGCTG
 TGTGCTGGAGACACTCGGAGCGGCGGGCCCCAGGCAAACCTGCACGACGCCTGCCA
 25 GGGCGATTTCGGGAGGCCCCCTGGTGTGTCTGAACGATGGCCGCATGACTTTGGTGGG
 CATCATCAGCTGGGGCCTGGGCTGTGGACAGAAGGATGTCCCGGGTGTGTACACAA
 AGGTTACCAACTACCTAGACTGGATTCGTGACAACATGCGACCGTGA (SEQ ID NO:7).

25. Fusion protein of OmpA and K2S, characterised in that it comprises a protein characterized
 by the following amino acid sequence or a fragment, a functional variant, an allelic variant, a
 30 subunit, a chemical derivative or a glycosylation variant thereof:

MKKTAIAIAVALAGFATVAQAASEGNSDCYFGNGSA YRGTHSLTESGASCLPWNSMILI
 GKVYTAQNPSAQUALGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLR

QYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERF
 PPHHLTVILGR TYRVVPGE EEQKFEVEKYIVHKEFDDDTYDNDIAL LQLKSDSSRCAQES
 SVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLL
 NRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQ
 5 KDVPGVYTKVTNYLDWIRDNM RPG (SEQ ID NO:8).

26. Fusion protein of OmpA and K2S according to claim 25, characterised in that it consists of a protein characterized by the following amino acid sequence:

MKKTALAI AVALAGFATVAQAASEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILI
 GKVYTAQNPSAQA LGLGKHNYCRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLR
 10 QYSQPQFRIKGGLFADIASHPWQAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERF
 PPHHLTVILGR TYRVVPGE EEQKFEVEKYIVHKEFDDDTYDNDIAL LQLKSDSSRCAQES
 SVVRTVCLPPADLQLPDWTECELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLL
 NRTVTDNMLCAGDTRSGGPQANLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQ
 KDVPGVYTKVTNYLDWIRDNM RPG (SEQ ID NO:8).

15 27. K2S protein, characterised in that it comprises a protein defined by the sequence SEGN (SEQ ID NO:9) and a or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

28. K2S protein according to claim 27, characterised in that it comprises a protein defined by the
 20 sequence SEGNSD (SEQ ID NO:10) and a or a variant or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative, a fusion protein or a glycosylation variant thereof.

29. K2S protein according to claim 28 or 29, characterised in that it comprises a protein characterized by the following amino acid sequence or a fragment, a functional variant, an allelic variant, a subunit, a chemical derivative or a glycosylation variant thereof:

25 SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQA LGLGKHNY
 CRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW
 QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTVILGR TYRVVPGE EEQ
 KFEVEKYIVHKEFDDDTYDNDIAL LQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC
 ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA
 30 NLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNM
 RP* (SEQ ID NO:11).

30. K2S according to any one of claims 27 to 30, characterised in that it consists of a protein characterized by the following amino acid sequence:

SEGNSDCYFGNGSAYRGTHSLTESGASCLPWNSMILIGKVYTAQNPSAQALGLGKHNY
 CRNPDGDAKPWCHVLKNRRLTWEYCDVPSCSTCGLRQYSQPQFRIKGGLFADIASHPW
 5 QAAIFAKHRRSPGERFLCGGILISSCWILSAAHCFQERFPPHHLTIVLGRTYRVVPGEEEQ
 KFEVEKYIVHKEFDDDTYDNDIALQLKSDSSRCAQESSVVRTVCLPPADLQLPDWTEC
 ELSGYGKHEALSPFYSERLKEAHVRLYPSSRCTSQHLLNRTVTDNMLCAGDTRSGGPQA
 NLHDACQGDSGGPLVCLNDGRMTLVGIISWGLGCGQKDVPGVYTKVTNYLDWIRDNM
 RP* (SEQ ID NO:11).

10 31. A vector containing a DNA sequence according to any one of claims 14 to 24.

32. A vector according to claim 31, wherein said DNA sequence is preceded by a lac promoter and a ribosomal binding site.

33. The vector pComb3HSS containing a DNA according to any one of claims 14 to 24, wherein the expression of the gp III protein is suppressed or inhibited by deleting the DNA molecule
 15 encoding said gp III protein or by a stop codon between the gene coding for a polypeptide containing the kringle 2 domain and the serine protease domain of tissue plasminogen activator protein and the protein III gene.

34. A prokaryotic host cell comprising a DNA molecule according to any one of claims 14 to 24.

35. A prokaryotic host cell comprising a vector according to any one of claims 31 to 33.

20 36. An E. coli host cell comprising a DNA molecule according to any one of claims 14 to 24.

37. An E. coli host cell comprising a vector according to any one of claims 31 to 33.

38. Use of a DNA molecule according to any one of claims 14 to 24 or of a vector according to any one of claims 31 to 33 or a host cell according to any one of claims 34 to 37 in a method for the production of a polypeptide having the activity of tissue plasminogen activator.

25 39. Use according to claim 38, wherein said method is a method according to any one of claims 1 to 13.

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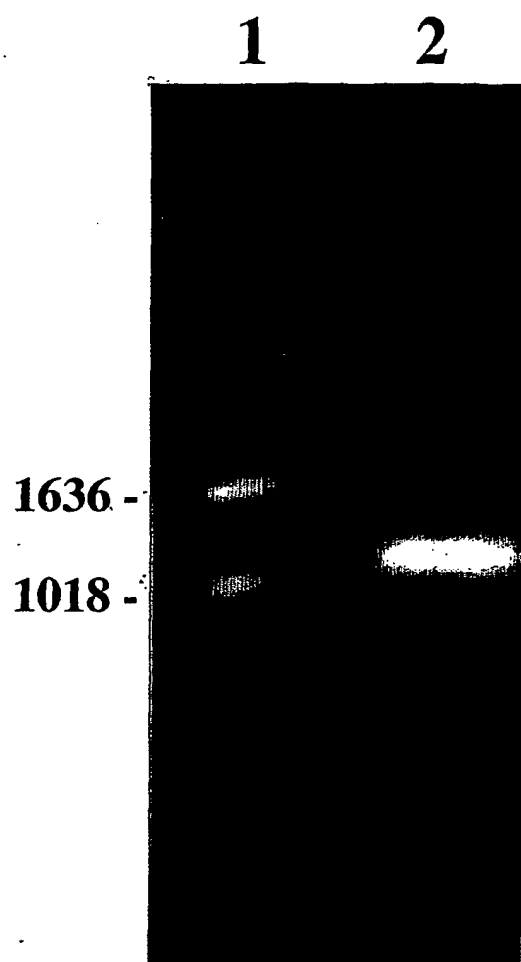


Fig. 1

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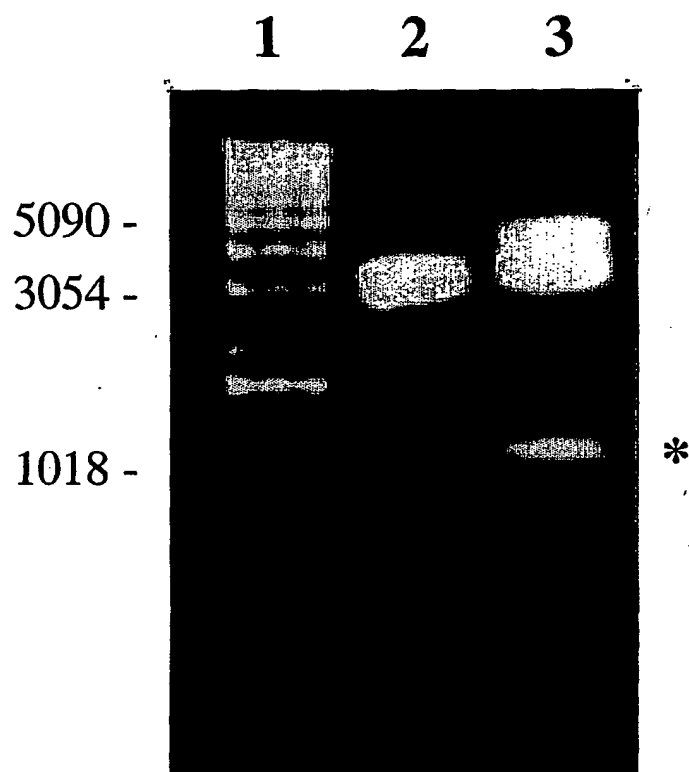


Fig. 2

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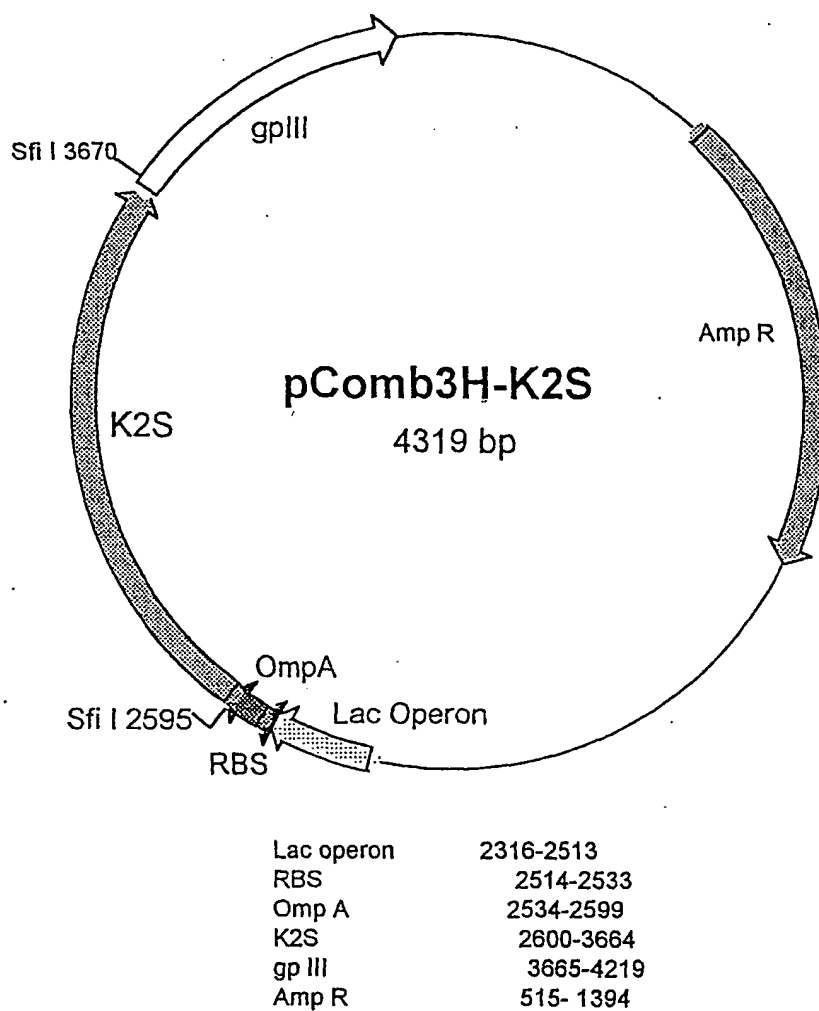


Figure 3

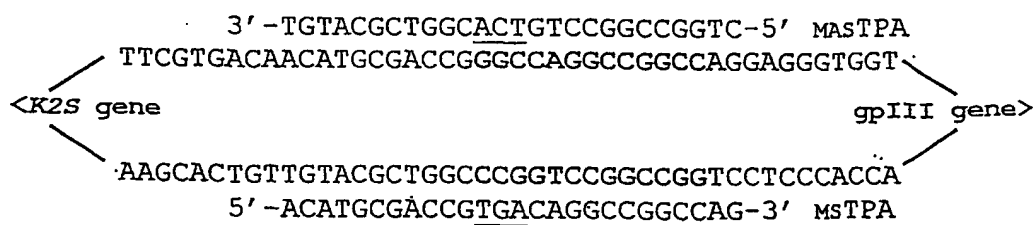


Figure 4

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5090 -

3054 -

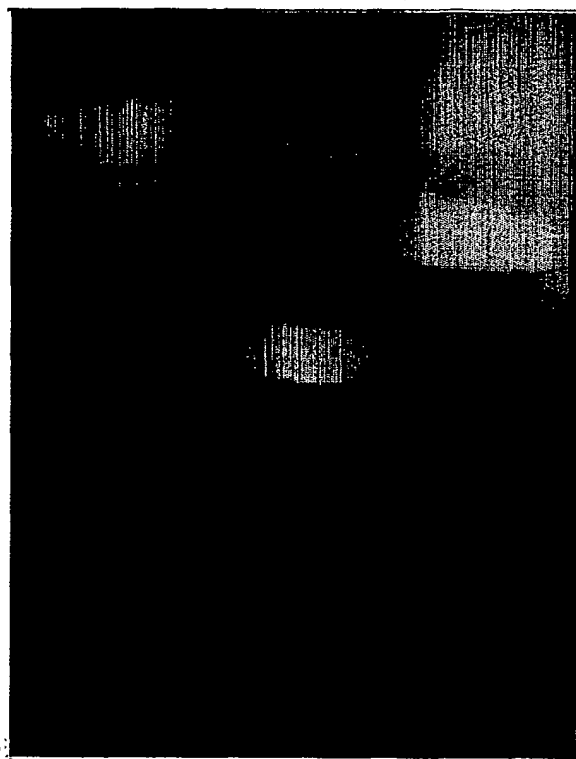


Fig. 5

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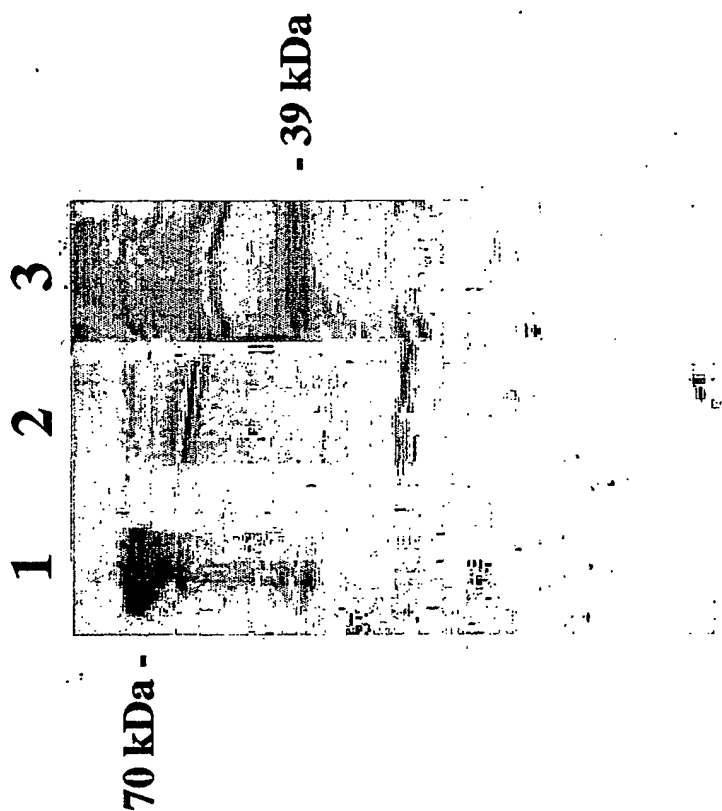


Fig. 6

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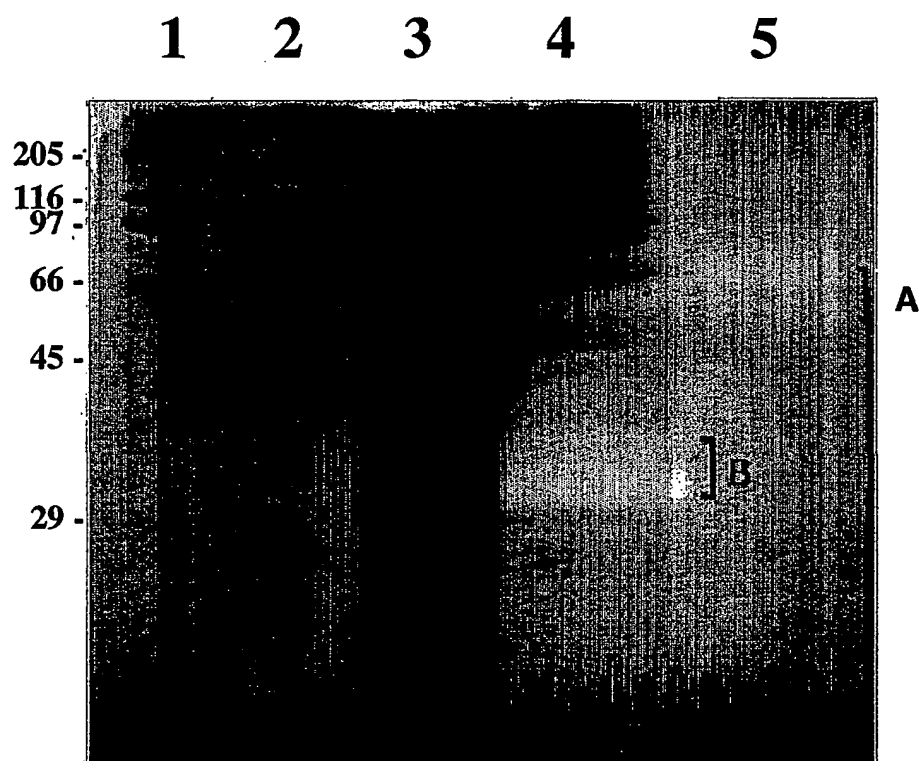


Fig. 7

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Fig. 8

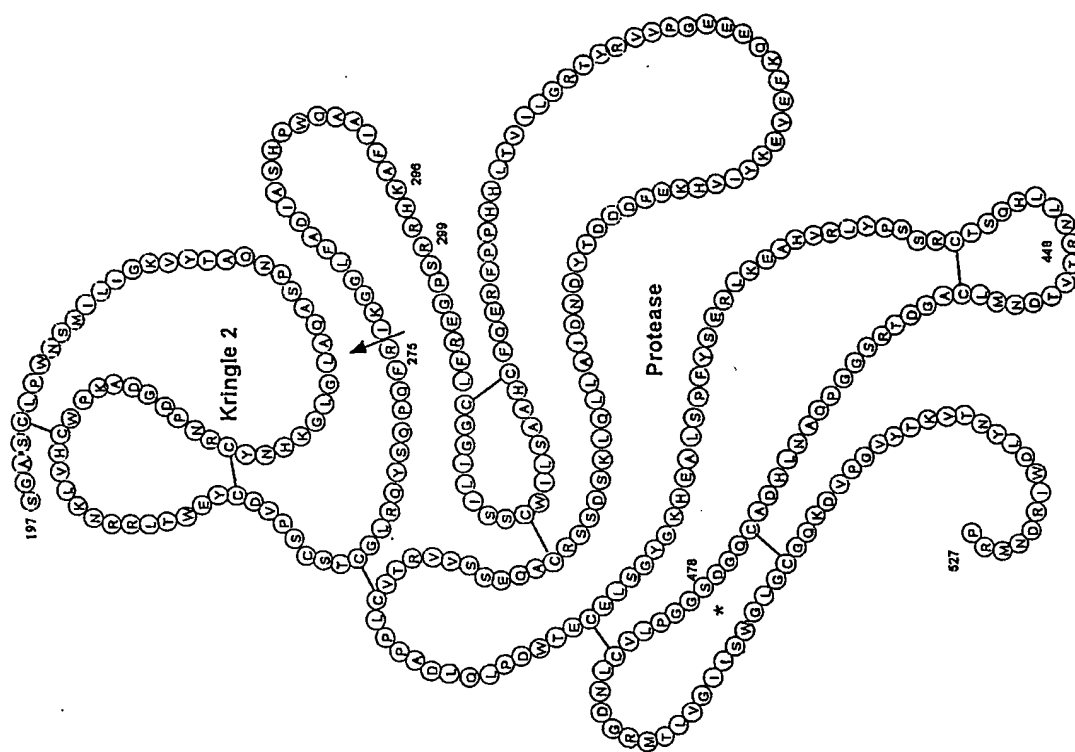


Fig. 9

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Fig. 10

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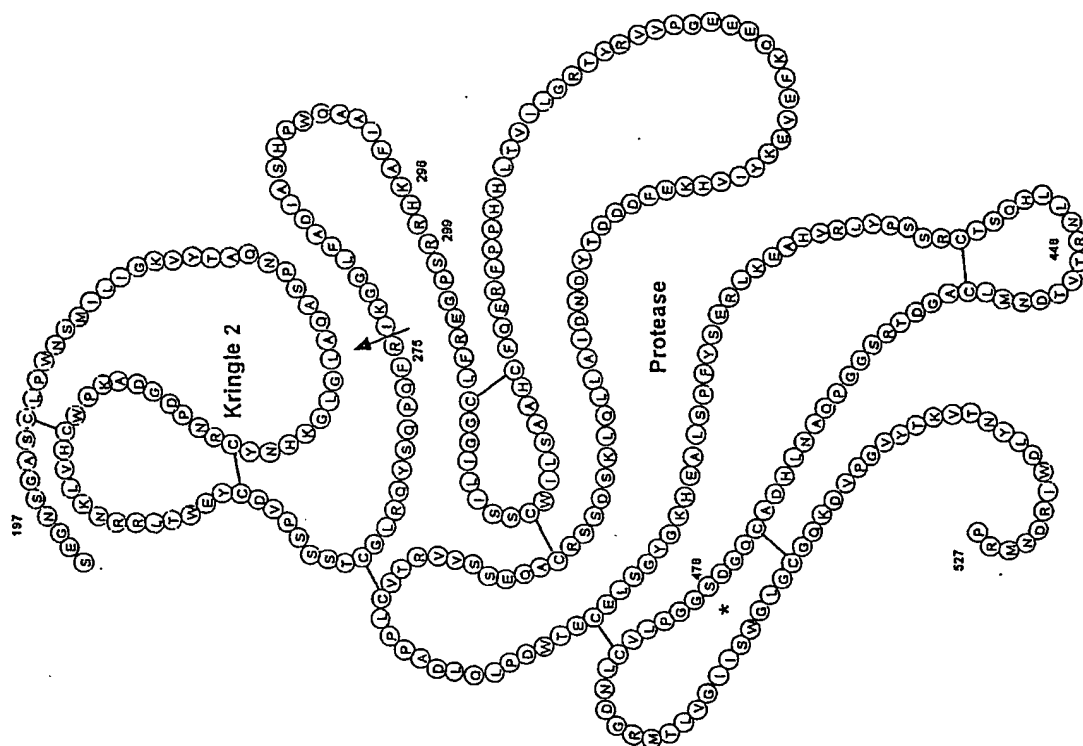


Fig. 11

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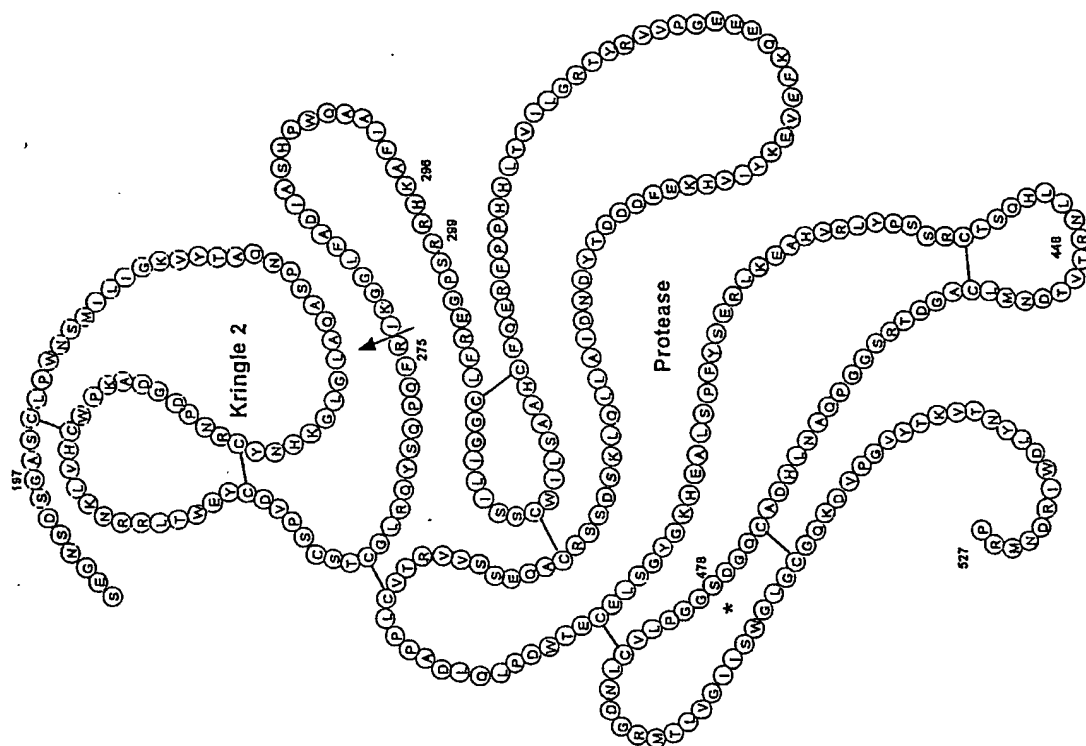


Fig. 12

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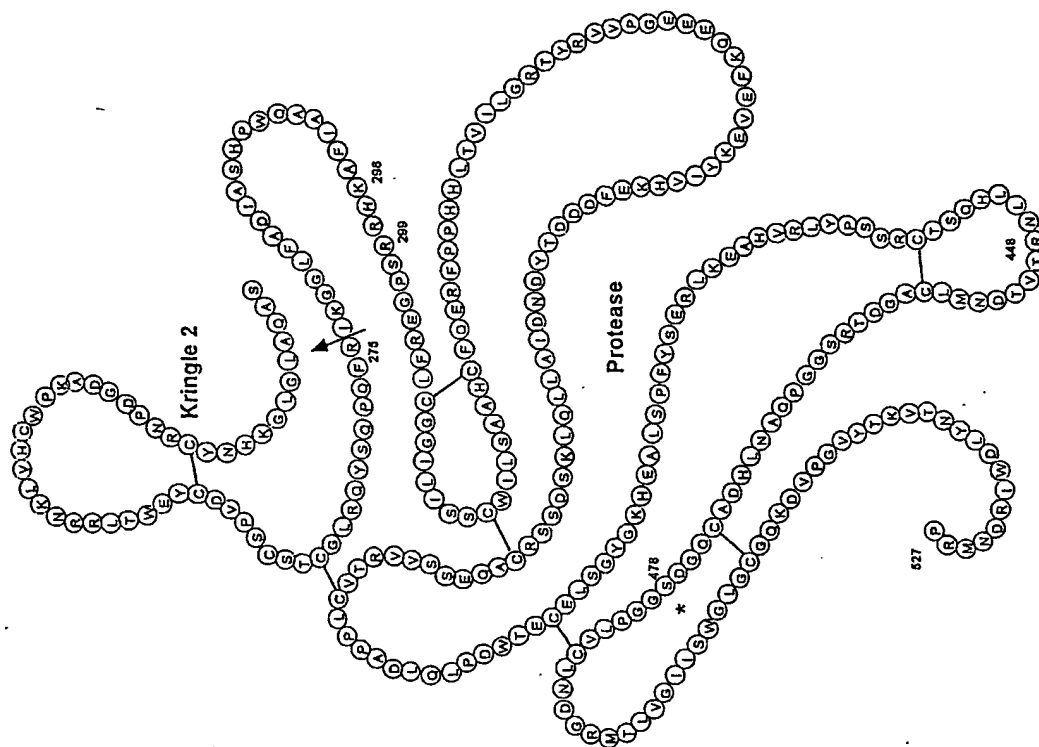


Fig. 14

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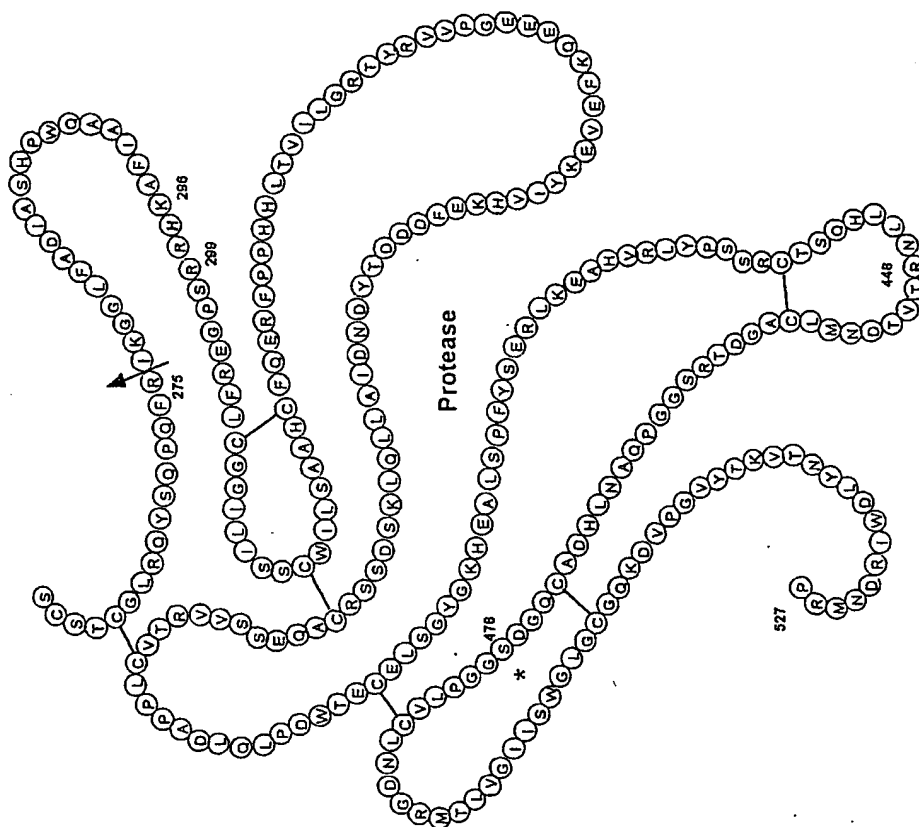


Fig. 15

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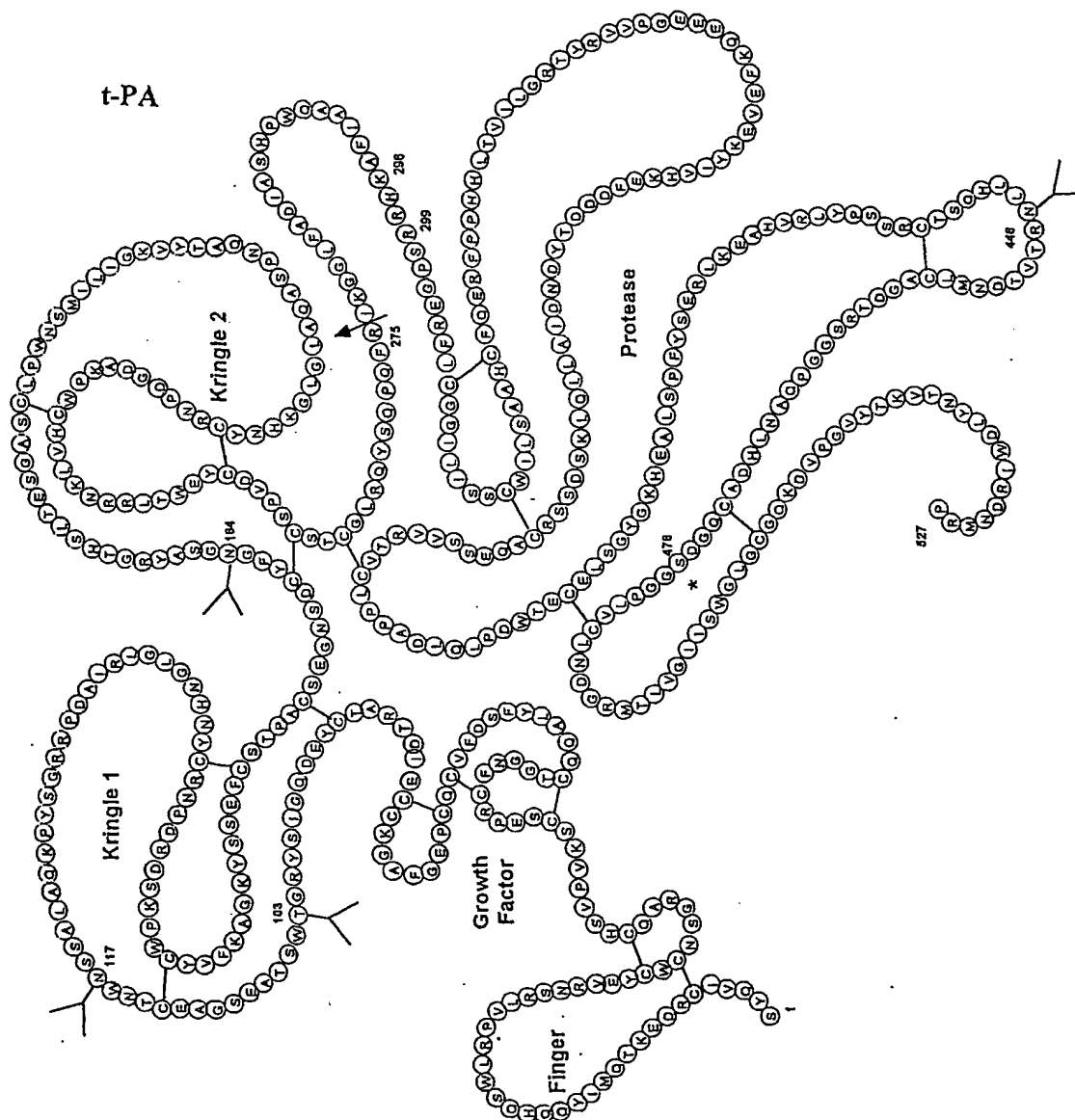


Fig. 16

SEQUENCE LISTING

<110> Boehringer Ingelheim International GmbH

<120> Methods for large scale production of recombinant
DNA-derived tPA or K2S molecules

<130> case 1-1170

<140>

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<150> GB 0027779.8

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<160> 25

<170> PatentIn Ver. 2.1

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<211> 1128

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<213> Artificial Sequence

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sequence for OmpA-K2S fusion protein

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30 <213> Escherichia coli

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<211> 1065

<212> DNA

<213> Artificial Sequence

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sequence for K2S protein

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10 tacacagcac agaaccacag tgcccaggca ctgggcctgg gaaacataa ttactgccgg 180
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<223> Description of Artificial Sequence: coding
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1

5

10

15

Thr Val Ala Gln Ala Ala Ser Glu Gly Asn Ser Asp Cys Tyr Phe Gly

35

20

25

30

Asn Gly Ser Ala Tyr Arg Gly Thr His Ser Leu Thr Glu Ser Gly Ala

35

40

45

Ser Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr
 50 55 60

Ala Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr
 65 70 75 80

Cys Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys
 85 90 95

10 Asn Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr
 100 105 110

Cys Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly
 115 120 125

15 Leu Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala
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Lys His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu
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Ile Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg
 165 170 175

25 Phe Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val
 180 185 190

Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val
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30 His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu
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Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val
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Arg Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr
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Glu Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe
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Tyr Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser
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Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met
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5

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15

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30

Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala

35

40

45

Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly

50

55

60

Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp

65

70

75

80

Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr

	85	90	95
	Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala		
	100	105	110
5	Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser Pro		
	115	120	125
	Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile		
10	130	135	140
	Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu		
	145	150	155 160
15	Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu		
	165	170	175
	Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp Asp		
	180	185	190
20	Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser		
	195	200	205
	Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu Pro		
25	210	215	220
	Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly		
	225	230	235 240
30	Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys		
	245	250	255
	Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His		
	260	265	270
35	Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp Thr		
	275	280	285
	Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly Asp		

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290 295 300

Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu Val
 305 310 315 320

5 Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro Gly
 325 330 335

Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met
 10 340 345 350

Arg Pro

15 <210> 12
 <211> 331
 <212> PRT
 <213> Artificial Sequence

20 <220>
 <223> Description of Artificial Sequence: K2S 197-527

<400> 12

25 Ser Gly Ala Ser Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys
 1 5 10 15

Val Tyr Thr Ala Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys
 20 25 30

30 His Asn Tyr Cys Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His
 35 40 45

Val Leu Lys Asn Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser
 35 50 55 60

Cys Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile
 65 70 75 80

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Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala
85 90 95

Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly
5 100 105 110

Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe
115 120 125

10 Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr
130 135 140

Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys
145 150 155 160

15 Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile
165 170 175

Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser
20 180 185 190

Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro
195 200 205

25 Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu
210 215 220

Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr
225 230 235 240

30 Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr
245 250 255

Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala
35 260 265 270

Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys
275 280 285

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Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu
290 295 300

Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn
305 310 315 320

Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro
325 330

<210> 13

<211> 339

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: K2S 193-527,
modified

<400> 13

Ser Glu Gly Asn Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp
1 5 10 15

Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser
20 25 30

Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp
35 40 45

Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr
50 55 60

Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln
65 70 75 80

Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile
85 90 95

Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser

100 105 110

Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp
115 120 125

5 Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His
130 135 140

Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu
10 145 150 155 160

Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp
165 170 175

15 Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp
180 185 190

Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu
195 200 205

20 Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser
210 215 220

Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu
25 225 230 235 240

Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln
245 250 255

30 His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp
260 265 270

Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly
275 280 285

35 Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu
290 295 300

Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro

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[illegible]

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<210> 14
<211> 335
<212> PRT
<213> Artificial Sequence
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<220>
<223> Description of Artificial Sequence: K2S 193-527,
modified

<400> 14

Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp Asn Ser Met Ile
1 5 10 15

Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala Gln Ala Leu
20 25 30

Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly Asp Ala Lys
35 40 45

Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp Glu Tyr Cys
50 55 60

Asp Val Pro Ser Ser Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro
65 70 75 80

Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro
85 90 95

Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg
100 105 110

Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala
 115 120 125

Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile
 130 135 140

Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe
 145 150 155 160

Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr
 165 170 175

Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys
 180 185 190

Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp
 195 200 205

Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys
 210 215 220

His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His
 225 230 235 240

Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn
 245 250 255

Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly
 260 265 270

Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly
 275 280 285

Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile
 290 295 300

Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr
 305 310 315 320

Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro
 325 330 335

<210> 15

<211> 343

<212> PRT

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: K2S 191-527,
 modified

<400> 15

Ser Glu Gly Asn Ser Asp Thr His Ser Leu Thr Glu Ser Gly Ala Ser
 1 5 10 15

Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala
 20 25 30

Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys
 35 40 45

Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn
 50 55 60

Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys
 65 70 75 80

Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu
 85 90 95

Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys
 100 105 110

His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile
 115 120 125

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Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe
130 135 140

Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val
145 150 155 160

Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His
165 170 175

10 Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln
180 185 190

Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg
195 200 205

15 Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu
210 215 220

Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr
20 225 230 235 240

Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg
245 250 255

25 Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu
260 265 270

Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp
275 280 285

30 Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly
290 295 300

Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln
35 305 310 315 320

Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp
325 330 335

Ile Arg Asp Asn Met Arg Pro
340

5 <210> 16

<211> 343

<212> PRT

<213> Artificial Sequence

10 <220>

<223> Description of Artificial Sequence: K2S 191-527,
modified

<400> 16

15 Ser Glu Gly Asn Ser Asp Thr His Ser Leu Thr Glu Ser Gly Ala Ser
1 5 10 15

Cys Leu Pro Trp Asn Ser Met Ile Leu Ile Gly Lys Val Tyr Thr Ala
20 25 30

20 Gln Asn Pro Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys
35 40 45

Arg Asn Pro Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn
25 50 55 60

Arg Arg Leu Thr Trp Glu Tyr Cys Asp Val Pro Ser Ser Ser Thr Cys
65 70 75 80

30 Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu
85 90 95

Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys
100 105 110

35 His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile
115 120 125

Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe

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130 135 140
 Pro Pro His His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val
 145 150 155 160
 5 Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His
 165 170 175
 Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln
 10 180 185 190
 Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg
 195 200 205
 15 Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu
 210 215 220
 Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr
 225 230 235 240
 20 Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg
 245 250 255
 Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu
 25 260 265 270
 Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp
 275 280 285
 30 Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly
 290 295 300
 Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln
 305 310 315 320
 35 Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp
 325 330 335
 Ile Arg Asp Asn Met Arg Pro

340

<210> 17

<211> 308

<212> PRT

<213> Artificial Sequence

<220>

10 <223> Description of Artificial Sequence: K2S 220-527

<400> 17

Ser Ala Gln Ala Leu Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro

1 5 10 15

15

Asp Gly Asp Ala Lys Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu

20 25 30

Thr Trp Glu Tyr Cys Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg

20 35 40 45

Gln Tyr Ser Gln Pro Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp

50 55 60

25 Ile Ala Ser His Pro Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg

65 70 75 80

Ser Pro Gly Glu Arg Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys

85 90 95

30

Trp Ile Leu Ser Ala Ala His Cys Phe Gln Glu Arg Phe Pro Pro His

100 105 110

His Leu Thr Val Ile Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu

35 115 120 125

Glu Glu Gln Lys Phe Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe

130 135 140

Asp Asp Asp Thr Tyr Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser
145 150 155 160

Asp Ser Ser Arg Cys Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys
165 170 175

Leu Pro Pro Ala Asp Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu
180 185 190

Ser Gly Tyr Gly Lys His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg
195 200 205

Leu Lys Glu Ala His Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser
210 215 220

Gln His Leu Leu Asn Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly
225 230 235 240

Asp Thr Arg Ser Gly Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln
245 250 255

Gly Asp Ser Gly Gly Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr
260 265 270

Leu Val Gly Ile Ile Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val
275 280 285

Pro Gly Val Tyr Thr Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp
290 295 300

Asn Met Arg Pro
305

<210> 18
<211> 268
<212> PRT
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: K2S 260-527

<400> 18

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5 Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro Gln Phe Arg
   1             5             10             15

Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro Trp Gln Ala
   20             25             30

10 Ala Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg Phe Leu Cys
   35             40             45

Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala Ala His Cys
15   50             55             60

Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile Leu Gly Arg
   65             70             75             80

20 Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe Glu Val Glu
   85             90             95

Lys Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr Asp Asn Asp
   100            105            110

25 Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys Ala Gln Glu
   115            120            125

Ser Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp Leu Gln Leu
30   130            135            140

Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys His Glu Ala
   145            150            155            160

35 Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His Val Arg Leu
   165            170            175

Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn Arg Thr Val
   180            185            190

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Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly Gly Pro Gln
 195 200 205

Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly Pro Leu Val
 210 215 220

Cys Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile Ser Trp Gly
 225 230 235 240

Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr Lys Val Thr
 245 250 255

Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro
 260 265

<210> 19
 <211> 527
 <212> PRT
 <213> Homo sapiens

<400> 19
 Ser Tyr Gln Val Ile Cys Arg Asp Glu Lys Thr Gln Met Ile Tyr Gln
 1 5 10 15

Gln His Gln Ser Trp Leu Arg Pro Val Leu Arg Ser Asn Arg Val Glu
 20 25 30

Tyr Cys Trp Cys Asn Ser Gly Arg Ala Gln Cys His Ser Val Pro Val
 35 40 45

Lys Ser Cys Ser Glu Pro Arg Cys Phe Asn Gly Gly Thr Cys Gln Gln
 50 55 60

Ala Leu Tyr Phe Ser Asp Phe Val Cys Gln Cys Pro Glu Gly Phe Ala
 65 70 75 80

Gly Lys Cys Cys Glu Ile Asp Thr Arg Ala Thr Cys Tyr Glu Asp Gln

	85	90	95
Gly Ile Ser Tyr Arg Gly Thr Trp Ser Thr Ala Glu Ser Gly Ala Glu	100	105	110
Cys Thr Asn Trp Asn Ser Ser Ala Leu Ala Gln Lys Pro Tyr Ser Gly	115	120	125
Arg Arg Pro Asp Ala Ile Arg Leu Gly Leu Gly Asn His Asn Tyr Cys	130	135	140
Arg Asn Pro Asp Arg Asp Ser Lys Pro Trp Cys Tyr Val Phe Lys Ala	145	150	155
Gly Lys Tyr Ser Ser Glu Phe Cys Ser Thr Pro Ala Cys Ser Glu Gly	165	170	175
Asn Ser Asp Cys Tyr Phe Gly Asn Gly Ser Ala Tyr Arg Gly Thr His	180	185	190
Ser Leu Thr Glu Ser Gly Ala Ser Cys Leu Pro Trp Asn Ser Met Ile	195	200	205
Leu Ile Gly Lys Val Tyr Thr Ala Gln Asn Pro Ser Ala Gln Ala Leu	210	215	220
Gly Leu Gly Lys His Asn Tyr Cys Arg Asn Pro Asp Gly Asp Ala Lys	225	230	235
Pro Trp Cys His Val Leu Lys Asn Arg Arg Leu Thr Trp Glu Tyr Cys	245	250	255
Asp Val Pro Ser Cys Ser Thr Cys Gly Leu Arg Gln Tyr Ser Gln Pro	260	265	270
Gln Phe Arg Ile Lys Gly Gly Leu Phe Ala Asp Ile Ala Ser His Pro	275	280	285
Trp Gln Ala Ala Ile Phe Ala Lys His Arg Arg Ser Pro Gly Glu Arg			

290 295 300

Phe Leu Cys Gly Gly Ile Leu Ile Ser Ser Cys Trp Ile Leu Ser Ala
305 310 315 320

5 Ala His Cys Phe Gln Glu Arg Phe Pro Pro His His Leu Thr Val Ile
325 330 335

Leu Gly Arg Thr Tyr Arg Val Val Pro Gly Glu Glu Glu Gln Lys Phe
10 340 345 350

Glu Val Glu Lys Tyr Ile Val His Lys Glu Phe Asp Asp Asp Thr Tyr
355 360 365

15 Asp Asn Asp Ile Ala Leu Leu Gln Leu Lys Ser Asp Ser Ser Arg Cys
370 375 380

Ala Gln Glu Ser Ser Val Val Arg Thr Val Cys Leu Pro Pro Ala Asp
385 390 395 400

20 Leu Gln Leu Pro Asp Trp Thr Glu Cys Glu Leu Ser Gly Tyr Gly Lys
405 410 415

His Glu Ala Leu Ser Pro Phe Tyr Ser Glu Arg Leu Lys Glu Ala His
25 420 425 430

Val Arg Leu Tyr Pro Ser Ser Arg Cys Thr Ser Gln His Leu Leu Asn
435 440 445

30 Arg Thr Val Thr Asp Asn Met Leu Cys Ala Gly Asp Thr Arg Ser Gly
450 455 460

Gly Pro Gln Ala Asn Leu His Asp Ala Cys Gln Gly Asp Ser Gly Gly
465 470 475 480

35 Pro Leu Val Cys Leu Asn Asp Gly Arg Met Thr Leu Val Gly Ile Ile
485 490 495

Ser Trp Gly Leu Gly Cys Gly Gln Lys Asp Val Pro Gly Val Tyr Thr

500

505

510

Lys Val Thr Asn Tyr Leu Asp Trp Ile Arg Asp Asn Met Arg Pro

515

520

525

5

<210> 20

<211> 12

<212> DNA

10

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: coding
sequence for SEGN

15

<400> 20

tctgagggaa ac

12

20

<210> 21

<211> 22

<212> PRT

<213> Escherichia coli

25

<400> 21

Met Lys Lys Thr Ala Ile Ala Ile Ala Val Ala Leu Ala Gly Phe Ala

1

5

10

15

Thr Val Ala Gln Ala Ala

30

20

<210> 22

<211> 42

35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 22

gaggaggagg tggcccaggc ggcctctgag ggaaacagtg ac

42

<210> 23

<211> 42

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 23

gaggaggagc tggccggcct ggcccggtcg catgttgtca cg

42

<210> 24

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 24

acatgcgacc gtgacaggcc ggccag

26

<210> 25

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 25

ctggccggcc tgtcacggtc gcatgt

26